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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*: Previous research from this laboratory indicated that 1) the expression of Tropomyosin-1 (TM1), a microfilament-associated protein, is abolished from many human breast carcinoma cells, and; 2) that TM1 is an anti-oncogene. These data led to the hypothesis that TM1 plays an important role in mammary carcinogenesis. Our results show that TM1 is downregulated in breast tumors (Objective 1). We demonstrated that TM1 is a suppressor of the malignant growth phenotype of MDA MB 231 cells, indicating that TM1 is a general suppressor of the transformed growth by inducing anoikis (Objective 2). We have designed siRNAs to inhibit TM1 expression (Objective 3). To assess the structure-function relationship of tumor suppression by TM1, we constructed chimeric and variant TM1 proteins. By employing a variant TM1 that contains an amino terminal extension, we show that the amino terminal integrity is important for TM1-mediated tumor suppression (Objective 4). Our work illustrates the critical role of TM1 in maintaining normal growth of breast epithelial cells. Future work is directed at elucidating the mechanism of TM1-mediated suppression of breast cancer and determining whether TM1 would be a useful biomarker.

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Introduction

The presence of disorganized or poorly structured microfilaments is a prominent feature of many transformed cells. Disorganized cytoskeleton is thought to contribute to the malignant growth of cells. Microfilaments regulate cell division, cell motility and intracellular transport. However, the role of microfilament-associated proteins in neoplastic transformation remains largely unclear. Downregulation of microfilament-associated proteins, such as tropomyosins (TMs) is hypothesized to result in the formation of functionally aberrant microfilaments, thus contributing to the manifestation of malignant cells. TMs are a family of cytoskeletal proteins that bind to and stabilize actin. This research proposal is based on the preliminary results which have identified that: 1) Tropomyosin-1 (TM1) is a suppressor of the transformed phenotype, and; 2) TM1 is consistently abolished in a large number of breast carcinoma cells that are tested. The main objectives of the proposed research are to assess the expression of TM1 in the tissue specimens of breast cancer patients and to investigate whether TM1 functions as a suppressor of the malignant growth of breast cancer. We have accomplished the proposed goals of the project.

Final Report

Technical Objective 1: Analysis of TM1 expression in human malignant breast tumors and benign lesions, and normal breast tissues:

Normal mammary epithelial cells elaborate multiple isoforms of TMs [1, 2]. For example. MCF10A cells express 7 different TM isoforms, including TM1 isoform which is the focus of our research (Figure 1A). The spontaneously transformed breast cancer cells exhibit severe deficiency in TM expression, with the expression of several TMs is either downregulated or completely abolished (Figure 1). For instance, TM38 protein is expressed in MDA MB231 cells, but found to be absent in MCF-7 cells. Expression of TM1, however, was consistently lacking in the breast carcinoma cell lines tested, indicating that TM1 suppression could be a common event during mammary carcinogenesis (Figure 1B). Based on these data, we hypothesized that loss of TM1 is a critical biochemical change in the malignant transformation of breast epithelial cells and that TM1 could be used as a novel biomarker of breast cancer.

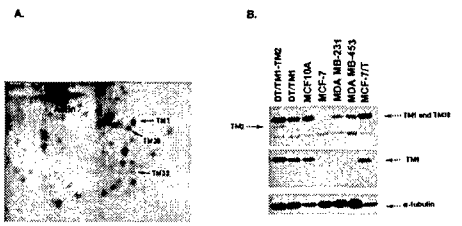


Figure 1. Expression of TMs in normal and breast cancer cells: A. Two dimensional gel analysis of TM expression in MCF10A cells. The positions of TM1, TM38 and TM32 are identified.

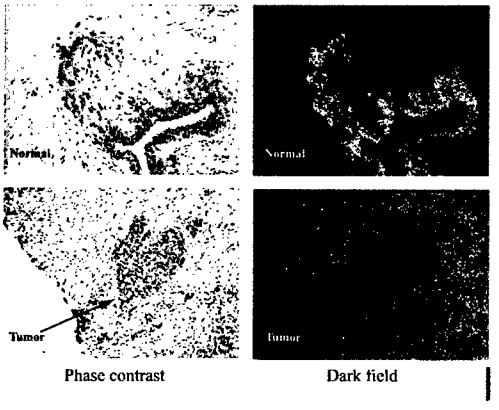
B. TM expression in normal and malignant breast cells. The cell lines used are indicated. DT/TM1 and DT/TM1-TM2 cells were used as positive controls for TM expression. These are TM1-induced revertants of ras-transformed fibroblasts expressing either TM1 alone or both TM1 and TM2. TM2 migrates as a distinct band below TM1, which is evident in DT/TM1-TM2 cells (top panel). The cell lysates were probed with either a polyclonal antiserum that reacts to multiple TMs (top panel), an anti-peptide antibody that reacts to TM1 specifically (middle panel), or with anti tubulin antibody for load controls (bottom panel). The anti-TM1 antibody recognizes TM1, but not other TM isoforms.

Since multiple TMs are present in epithelial cells, and the presence of stromal components (which abundantly express TM1) in tissues, we chose to analyze the tissues by immunohistochemistry. The smooth muscle cells of the blood vessels also express TM1. Currently available antisera recognize multiple isoforms of TMs and, thus, do not permit accurate analysis of TM1 expression. We have proposed to employ TM-specific antibodies to assess TM1 expression in breast tissues. We generated several TM1-specific antibodies in this lab. TM1-specific reactivity of these antibodies (Figure 1B) has been tested and described in two recent publications [2, 3]. For example, the MDA MB 231 and MDA MB 435 cells, which lack TM1 but express a co-migrating TM isoform (known as TM38), whereas some breast cancer cells (such as MCF-7) lack both TM1 and TM38. This is illustrated in blot that was probed with a pan TM antibody which recognizes multiple TMs, including TM1 and TM38 (Figure 1B top panel). The TM1-specific antibody (Figure 1B, middle panel) detects TM1 in normal MCF10A cells, but does not yield a signal in the breast cancer cells, independent of the presence of co-migrating TM38. This result is consistent with the 2-dimensional gel electrophoresis data [1]. The other fibroblast cell lines (DT/TM1 and DT/TM1-TM2) in the figure are used as references which express either TM1 [4] or TM1 and TM2 [5]. These results demonstrate that the novel antibodies are highly TM1-specific.

In addition, we have developed a sensitive in situ hybridization method to determine TM1 expression in breast tissues. For this purpose, we collected normal and malignant breast

tissue specimens from the patients undergoing surgery, as part of their clinical care. Tissue specimens were collected under the approval of the Institutional Review Board. We analyzed 24 normal and 25 invasive breast tumors for TM1 expression. While all of the normal tissues expressed abundant quantities of TM1, none of the breast tumors expressed significant levels of TM1 (Figure 2A). Quantitation of TM1 expression revealed profound differences between

Figure 2A. TM expression in normal and malignant breast tissues:: Normal and malignant breast tissues were hybridized with antisense (top and bottom panels) TM1 probes. Samples were photographed in bright field to view the tissue architecture (left panels) and in dark field to view the silver grains indicative of TM1 expression (right panels). Samples were photographed at 25x magnification.



normal ducts and the invasive tumors. TM1 mRNA expression was quantitated by counting the intensity and number of the silver grains. Relative quantity of TM1 in normal tissue was at 29.7 units, while the malignant breast tissue expressed TM1 at 3.5 units (Figure 2B). The signal obtained with the tumors was comparable to that obtained with the 'sense' control probes. These data demonstrate that the expression of TM1 is profoundly suppressed in breast tumors [3].

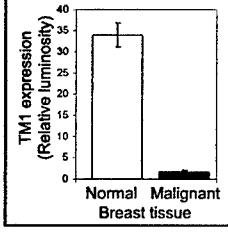


Figure 2B: The expression of TM1 mRNA is profoundly suppressed in breast tissues: TM1 expression was quantified by relative luminescence. The signal due to background was subtracted from that of normal and tumor samples.

In a second line of study to determine the changes in TM1 expression in breast tumors, we have employed immunofluorescence to determine changes in TM1 expression in breast tissues. We have employed the novel TM1 specific antibodies [2] to evaluate TM1 expression in breast tissues. Consistent with the results obtained with in situ hybridization data, TM1 protein was undetectable in 25 primary tumors, while all the normal tissues expressed TM1 (Figure 3) [3]. TM1 expression was noted in luminal and basal layers of normal ducts. The tumor tissue was negative for TM1 expression. It should be noted that the blood vessels and stromal elements express TM1 in significant amounts. The smooth muscle component of blood vessels expresses TM1, and accordingly, the TM1 antibody showed strong reactivity (identified with and arrow, Figure 3 middle, bottom panel). The reactivity of the antibody with the blood vessels serves as an internal positive control for TM1 expression in tumor tissues, which are negative for TM1.

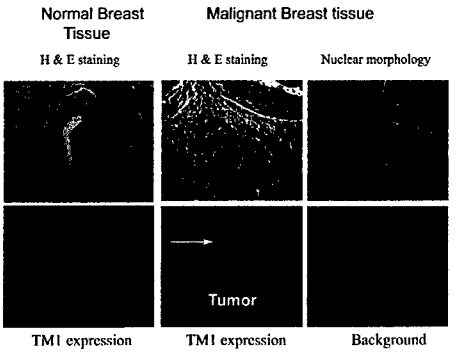


Figure 3. Downregulation of TM1 expression in breast tumors: TM1 expression was determined by immunofluorescence. The background indicates staining of the tissue in the absence of primary antibody, which did not yield any measurable signal. The samples were counterstained with DAPI to visualize nuclear morphology. TM1 was readily detected in normal ducts. The reactivity in tumor tissue is due to the smooth muscle cells present in the blood vessels (indicated with an arrow), but the tumor itself (labeled) is negative for TM1.

We next screened several commonly employed lung and colon carcinoma cell lines for TM1 expression to determine whether loss of TM1 expression is specific for breast cancer cell lines. These results indicated while some lung and colon carcinoma cells lack TM1, some contain detectable TM1, suggesting that the consistent loss of TM1 expression is a specific feature of breast carcinoma cells.

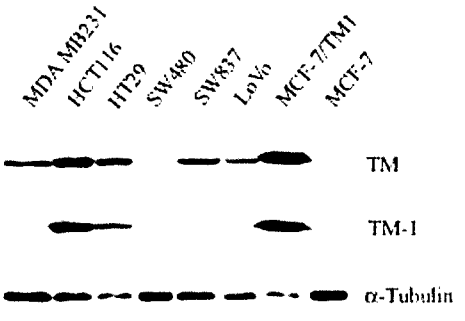


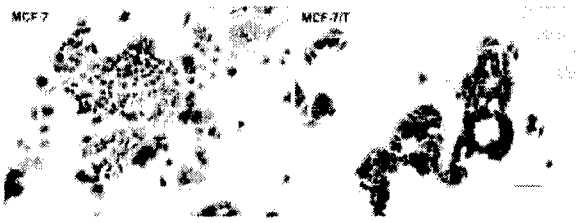
Figure 4. TM1 expression in breast and colon carcinoma cell lines: Cell lysates from indicated cell lines were probed with a TM1-specific antibody, a pan-TM antibody and α-tubulin antibody. MDA MB 231 and MCF-7 are breast cancer cell lines. MCF-7/TM1 is derived by transduction of MCF-7 cells with TM1, and it is a revertant cell line. The other cell lines are colon carcinoma cell lines. The panel labeled as 'TM' indicates immunoblotting with the pan TM antibody which recognizes TM1 and other TM isoforms.

Technical Objective 2: Effects of expression of TM1 in human breast carcinoma cell lines and in normal mammary epithelial cell lines.

Our previous studies with experimentally transformed murine fibroblasts have demonstrated that TM1 is a suppressor of the malignant transformation, and that TM1 is a class II tumor suppressor [2, 6, 7]. To examine the role of TM1 in mammary carcinogenesis, and to determine whether TM1 can suppress the malignant growth of a spontaneously transformed human breast carcinoma cells, the following experiments were carried out.

MCF-7 cells, which lack TM1, were transduced with a recombinant retrovirus expressing TM1. Individual cell lines expressing TM1 were isolated and tested for the effects of TM1 expression on the morphology and growth properties [2]. Restoration of TM1 expression resulted in the formation of tighter colonies with a more branched, tubular appearance (Figure 5A). Restoration of TM1 expression resulted in the emergence of TM1 containing microfilaments [2].

Figure 5A. . Morphology of TM1 expressing MCF-7 cells: Monolayers of MCF-7 and MCF-7 cells transduced with TM1 (MCF-7/T cells) were stained with H & E and photographed using an Olympus B20 microscope with 2x objective.



Transduction of MCF-7 cells resulted in marked changes in the growth of MCF-7 cells. TM1 expression significantly decreased the growth rates, compared to parental MCF-7 cells. A more profound effect was observed on the anchorage-independent growth property, which is a hall mark of the neoplastic phenotype (Figure 5B & C). TM1 expression completely abolished the anchorage independent growth of MCF-7 cells, indicating that TM1 suppressed the malignant findings support the hypothesis that TM1 is a general suppressor of neoplastic growth.control by estrogen. In a different approach, we have adenoviraly expressed TM1 in MCF-7 cells, which also resulted in suppression of the anchorage-independent growth [3]. It should be noted that the revertant MCF-T/T cells remain responsive to estrogen in their growth behavior. We have also demonstrated that restoration of TM1 expression results in increased association of cell adhesion molecules, E-cadherin and β–catenin with the cytoskeletal fraction, which may explain the formation of tighter colonies in MCF-7/T cells (Figure 5A) [2].

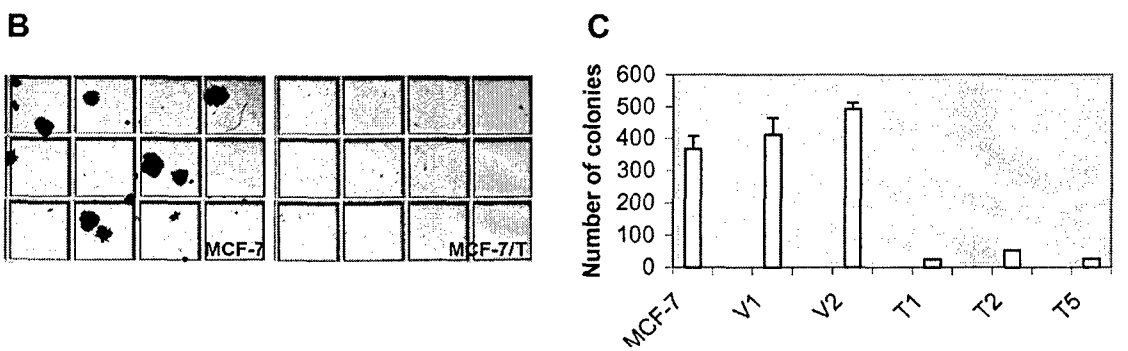


Figure 5B & C. TM1 suppresses anchorage independent growth: Cells were plated in soft agar as described in materials and methods. At the end of the culture, they were stained with nitroblue tetrazolium, photographed (B) and the number of colonies formed with each cell line is shown (C). Error bars indicate standard deviation.

With these experiments, the goals of this Objective are accomplished, and we have completed the Objective 2.

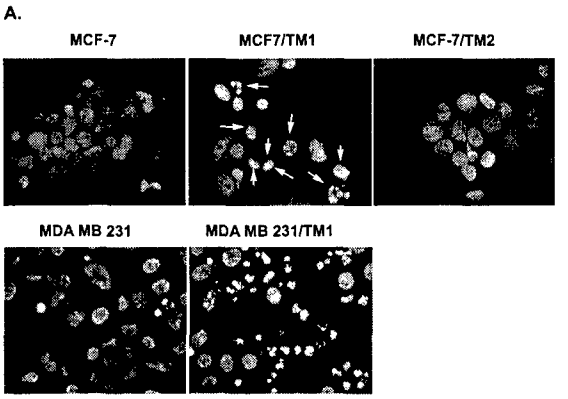
The following work represents additional studies on the role of cytoskeletal proteins in breast cancer related to this Objective, which were not propsed original grant application. We have made significant progress in understanding the role of TM1 in regulating growth and survival of normal and malignant breast cells. We have tested whether TM1-mediated tumor

suppression is cell type specific or whether TM1 suppresses the neoplastic behavior of other breast cancer cells, which differ in the hormone receptor or p53 status.

For this purpose, we selected MDA MB 231 cells, which lack TM1, but express another related TM isoform, viz., TM38 (Figure 1B). We have demonstrated that TM1 suppresses the malignant growth properties of MDA MB 231 cells [2]. Collectively, these results show that TM1 suppresses the malignant growth cells of breast cancer cells.

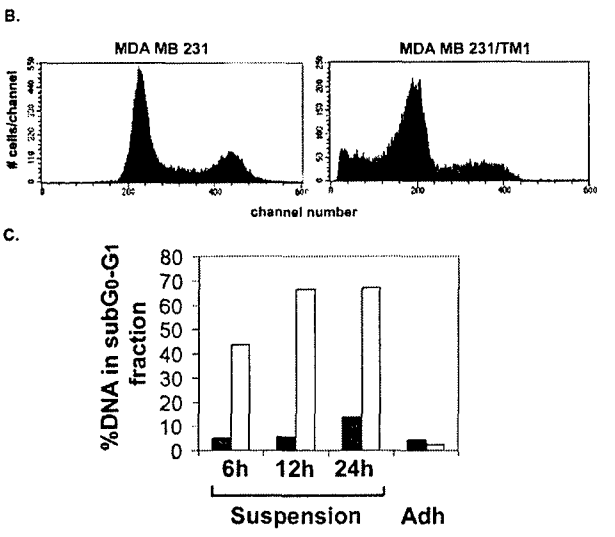
A new line of work to investigate the mechanism of tumor suppression by TM1 is initiated. Since TM1 suppresses anchorage independent growth, we investigated whether TM1 induces anoikis (detachment-induced apoptosis) in breast cancer cells (Figure 6). Initial studies

Figure 6A: TM1 induces anoikis in breast cancer cells: Parental MCF-7 and MDA MB 231 cells, and those express TM1 and TM2 were cultured on poly HEMA coated dishes for 72h (MCF-7 derived cells), or 24h (MDA MB 231 derived cells). A. Nuclear morphology of DAPI stained cells is shown. Apoptotic cells are marked with arrows in MCF-7/TM1 cell panel. Cells were photographed using a fluorescent microscope with a UV filter cube at 100x magnification. As shown in the figure, TM1-transduced breast cancer cells undergo apoptosis when denied adhesion.



indicate that TM1 sensitizes breast cancer cells to anoikis [3]. Detachment-induced apoptosis in TM1-transduced cells, but not in the parental breast cancer cells has been demonstrated by examining nuclear morphology (Figure 6A) and measuring the DNA content in subG_0-G_1 fraction of cell cycle (Figure 6B &C).

Figure 6B and C. TM1 expression induces rapid anoikis: Accumulation of DNA in sub G_0-G_1 fraction, which indicates apoptosis, was measured by propidium iodide staining. MDA MB 231 and MDA MB 231/TM1 expressing cells were cultured in suspension for 6 to 24 hours and harvested. Cell cycle distribution at six hours is shown in Figure 6 B. The percent DNA content in subG_0-G_1 fraction is shown (Figure 6C). Shaded boxes represent MDA MB 231 cells and the open boxes are MDA MB 231/TM1 cells. DNA content in subG_0-G_1 fraction of cells cultured under normal adhesion conditions (marked 'adh') has been quantitated. These samples do not contain any significant amount of apoptotic DNA.



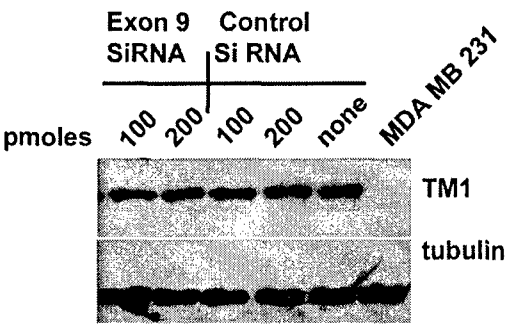
Ongoing work indicates that TM1 expression in MDA MB 231 cells alters integrin profiles, which may re-sensitize them to anoikis. We are now investigating whether TM1-induced anoikis is mediated through altered integrin-controlled intracellular signaling pathways [8].

Technical Objective 3. Induction of transformed phenotype by repression of TM1 expression: In order to test whether the loss of TM1 expression could lead to the expression of malignant transformation of mammary epithelium, antisense suppression of TM1 is proposed. TM1 was subcloned in antisense direction in the retroviral vector pBNC and antisense packaging cells of PA317 are generated. The MCF10A cells have been transduced with the recombinant pBNC retrovirus. Transduced cells were selected for resistance to G418 and single cell clones were selected. These cell lines were tested for growth under anchorage independent conditions. MCF10A cells expressing antisense TM1 were unable to grow under anchorage independent growth conditions, indicating that antisense expression was not adequate to suppress TM1 expression. These data indicate that either the antisense RNA is not effective in suppressing TM1 or, the antisense suppression of TM1 alone does not transform the breast cancer cells. This difficulty was foreseen in the potential problems section of the grant proposal. TM1 is a major cytoskeletal protein with presumably a longer half-life, which may contribute to resistance to the classical anti-sense-suprression. We have completed the work as proposed in the original application.

At this point, we have tested the siRNA strategy to suppress TM1 in MCF10A cells. We have identified a sequence in the exon 9 of TM1 to target the siRNAs. We have also employed complementary control siRNAs through commercial sources. The siRNAs have been transfected into MCF10A cells and the expression of TM1 was assessed by the isoform-specific antibodies by immunoblotting. As shown in the Figure 7, TM1 expression was not significantly suppressed in the siRNA-treated cells compared to the control siRNA-treated cells or unmodified MCF10A cells. Since we find the siRNA directed at the exon 9 is unable to suppress TM1 expression, we will test other potential targets to achieve the 'knock-down' of TM1.

Although our attempts to suppress TM1 hitherto have not succeeded, the complementary strategy of replacing TM1 in breast tumor cells (Objective 2) has been highly successful. Our work has shown for the first thime that TM1 is a suppressor of malignant phenotype of breast cancer cells.

Figure 7. Effect of exon 9 siRNA on TM1 expression in MCF10A cells: SiRNA targeting exon 9 of TM1 and a control siRNA were used to transfect MCF10A cells at 100 and 200 pmoles. The expression of TM1 was evaluated by immunoblotting using TM1-specific antibodies. Lysates from untransfected MCF10A cells (marked 'none') were used as controls. MDA MB 231 cells which lack TM1 were used as controls for the specificity of the TM antibody (Figure 1). The blot was also probed with anti-tubulin antibodies for load contols.



Technical Objective 4. Structure-function relationship of TM1-mediated tumor suppression: Studies completed in Techinical Objective 2 have shown that TM1 is a suppressor of malignant growth. Experiments proposed in this Objective were aimed at investigating the molecular basis of anti-neoplastic effects of TM1.

First, we tested the isoform-specificity of tumor suppression by TM1. For this purpose, we have created a cell line derived from MCF-7 cells that expresses TM2-an isoform that shares significant homology with TM1. We have subcloned TM2 cDNA into a plasmid vector that co-expresses green fluorescent protein via IRES sequences to mark the transfected cells [3]. MCF-7 cells were transfected with TM2 plasmid and selected with the G418. Stable cells expressing TM2 were tested for their growth in agar. TM2 cells grew in anchorage independent conditions,

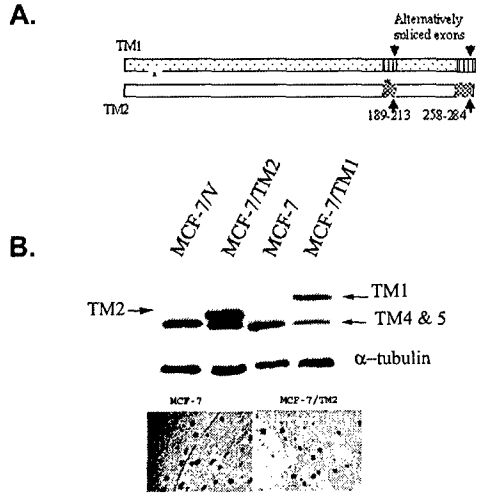


Figure 8: Isoform-specificity of tumor suppression by TM1. A. Comparison of TM1 and TM2 proteins. TM1 and TM2 share extensive sequence homology except in the sequence coded by two alternatively spliced exons (amino acids 189-213 and 258-284). The asterisks identify unique cysteine residues in TM1 (cys 35) and TM2 (cys 190).
B. TM2 is not a tumor suppressor of breast cancer cells: MCF-7 cells were transfected to restore the expression of TM2, and stably selected cells were tested for TM2 expression using a commercial antibody (TM311, Sigma). Unmodified MCF-7 cells, empty vector transduced cells (MCF-7/V), TM2 and TM1 expressing MCF-7 cells were used in the immunoblotting experiment (top). Parental and TM2 expressing MCF-7 cells were cultured under anchorage independent conditions (bottom).

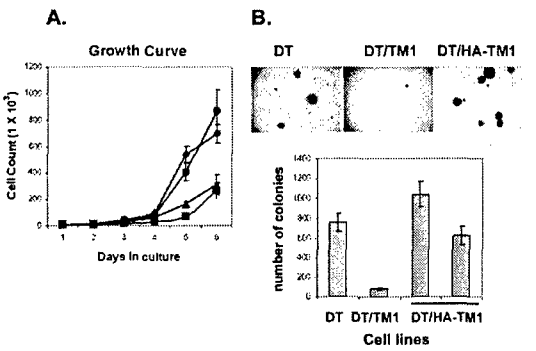
indicating that TM2, unlike TM1, is not a tumor suppressor (Figure 8).

At this point, we have taken two different approaches to determine the molecular determinants of TM1 that are responsible for tumor suppression. The terminal portions of TMs are known to be important regulators of TM functions. Since TMs are α-helical coiled-coil molecules, we have sought to modify the amino terminal end of TM1 by adding a hemagglutinin (HA) epitope. Epitope tagging of TM1 is expected to greatly facilitate the analysis of transfected TMs and proived insights into the functional importance of the termini of TM1. To confirm that epitope tagging does not impair the tumor suppressive function of TM1, we chose to test the constructs in DT cell (ras-transformed NIH3T3 cells) system. Our previous published studies showed that DT cells are suitable models to test the ability of TM1 to reorganize the cytoskeleton and tumor suppression. For example, the effects of TM1 on the cytoskeleton and growth of DT cells [4] are essentially similar to that observed with breast cancer cells [2]. Since the modified TMs exert effects beyond the cell morphology and anchorage-independent growth, we conducted these experiments in simpler, yet relevant model. For example, we found that the modified TMs alter cytokinesis, which renders the isolation of single cell clones difficult [9, 10]. The effects of TMs on cytokinesis was discovered during the project period and are further discussed in the accompanying publications [9, 10].

In the first approach, a hemagglutinin (HA) epitope was engineered to the amino terminus of TM1. The ATG initiation codon of TM1 was replaced with an oligonucleotide sequence encoding HA epitope. The recombinant TM1 was subcloned into a eukaryotic expression vector, and transfected into DT cells.

The epitope tagged TM1 profoundly differed from the wild type protein in terms of its

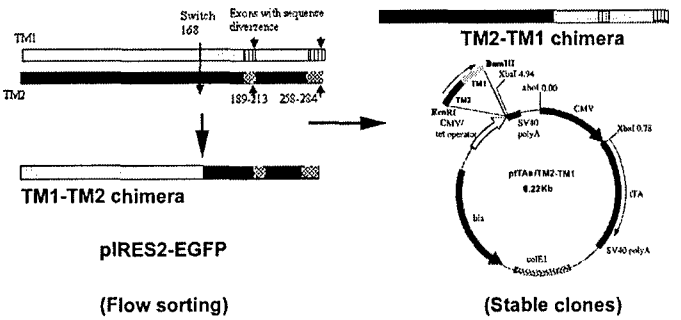
Figure 9. Amino terminal modification abolishes anti-oncogenic effects of TM1: Growth of cells in monolayer (A), or under anchorage independent conditions (B) was measured. In A, the following cell lines were used: DT/TM1 (■), NIH3T3 (▲), DT/HA-TM1 (◆) and DT (•). The p values were calculated using two tailed *t* test assuming unequal variance. Photomicrographs of anchorage independent cultures (B) are shown. The histogram shows the efficiency of colony formation in DT, two independent clones of DT/HA-TM1 and DT/TM1 cells. The error bars indicate mean ± standard deviation from triplicate samples.



ability to function as a tumor suppressor [9]. Transfection of epitope tagged TM1 did not induce microfilaments, alter the growth rates or inhibit the growth in agar (Figure 9). These data show that modification of N-terminal portion of TM1 completely abolishes the tumor suppression. These results further support the importance of the amino terminus of TM1 in cytoskeletal reorganization and suppression of malignant growth.

In the second approach, we created chimeras of TM1 and TM2 as described in Figure 10. To test whether the amino and carboxy terminal portions of TM1 are responsible for tumor suppression, chimeras of TM1 (a tumor suppressor) and TM2 (not a tumor suppressor) were created [10]. We have completed the site directed mutagenesis to introduce a silent mutation to create a HindIII restriction site. This was accomplished by PCR and the resultant variants of TM1 and TM2, designated as 'TM1-TM2 and 'TM2-TM1' respectively. We have tested the

Figure 10. Generation of TM chimeras: A schematic representation of TM1 and TM2 cDNAs with areas of sequence divergence, and the position at which the amino and carboxy termini are switched (designated as 'Switch') is depicted. The TM2-TM1 chimera is subcloned into a tetracycline regulatable plasmid vector, ptTAs that allows selection with puromycin. In this vector, the tetracycline regulated transactivator and the tetracycline-repressed CMV promoters are present. The expression of the cloned gene is repressed in the presence of the antibiotic. The TM1-TM2 chimera is cloned into pIRES2-EGFP plasmid, and the transfected cells are isolated by flow cyotometer.



anti-oncogenic effects of the chimeras DT cells.

We have used a tetracycline-inducible expression system to express the TM2-TM1 chimera (Figure 10) which contains the carboxy terminal portion of TM1. DT cells were transfected with TM2-TM1 chimera and single cell clones were isolated in presence of tetracycline which represses the expression of the transfected gene. The single cell clones were induced by withdrawing the antibiotic from the culture medium and the microfilament architecture and anchorage-independent growth was assessed (Figure 11). The TM2-TM1 chimera fails to induce stress fibers or suppress anchorage-independent growth [10]. This result was somewhat unexpected because the carboxyterminal portion of TM1 contains distinctive sequences hypothesized to contribute to the isoform-specific functions of TM1. However, the results of the experiments with the modified TM1 (Figure 9) and the TM2-TM1 chimera (Figure 11) indicate that the amino terminus may be important determinant of TM1 functions. This **new hypothesis** is tested in the next set of experiments.

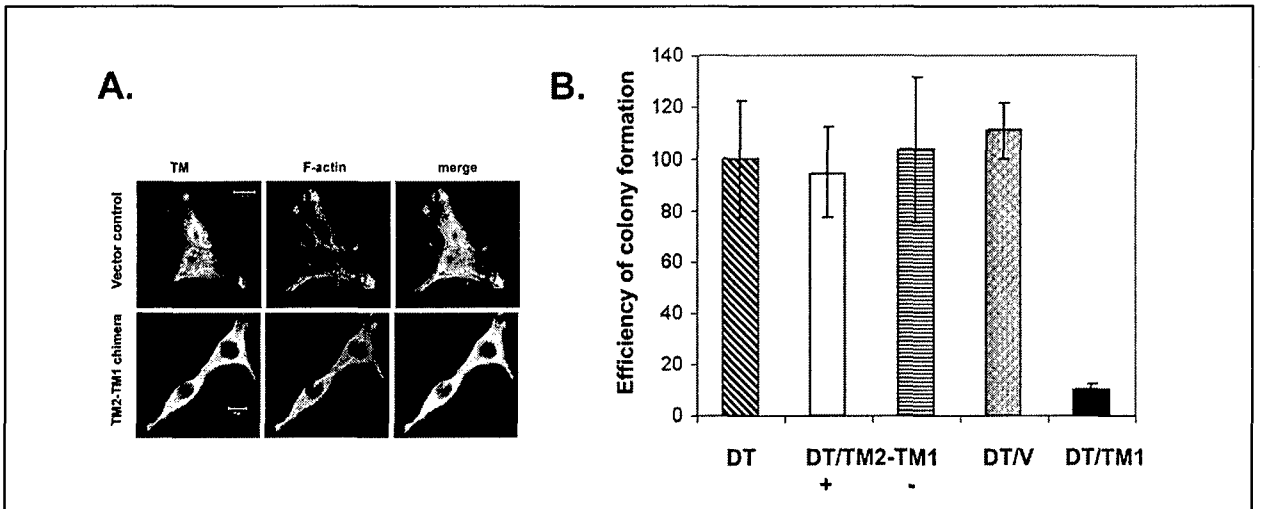


Figure 11: TM2-TM1 chimera does not assemble stress fibers or suppress anchorage-independent growth: ***A.*.** Immunofluorescence revealed that the induction of TM2-TM1 chimera fails to induce stress fibers, as revealed by TM antibody and F-actin (staining with phalloidin), and the cells resembled parental DT and DT/TM2 cells. Bar 10μm. ***B***. Expression of the TM2-TM1 chimera does not inhibit the anchorage independent growth. The DT/TM2-TM1 cell line was cultured either in the presence of tetracycline (+) or absence (-), which corresponds to uninduced and induced conditions, respectively. A vector control cell line (DT/V) was also plated which efficiently grew under anchorage-independent conditions. The number of colonies obtained is normalized to DT cells (100%). TM1 expression (DT/TM1 cells), abolishes the growth of DT cells and is included as a reference. Expression of TM2 in DT cells does not alter anchorage independent growth, and therefore, not depicted.

Expression of TM1-TM2 chimera which contains the amino terminal portion of TM1(Figure 10) induced stress fibers in DT cells (Figure 12). However, expression of the chimera resulted in extensive endoreduplication, which precluded the isolation of single cell clones for further study [10]. Thus, collectively our results show that the amino terminus is critical for TM1-induced cytoskeletal reorganization and suppression of malignant growth. Current efforts are now directed at further defining the 'domains' in the amino terminal region of TM1 that induce stress fibre formation and suppression of anchorage independent growth.

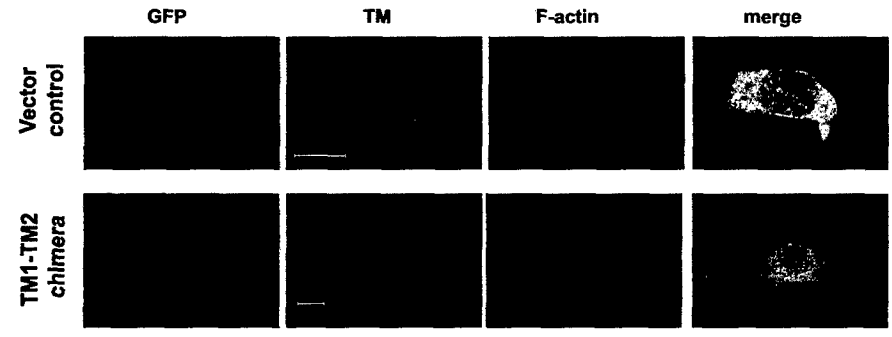


Figure 12. TM1-TM2 chimera induces stress fiber formation: ***A. B.*** DT cells transected with empty pIRES2-EGFP (vector control) and the chimera (TM1-TM2 chimera) cells were stained with TM311 antibody and phalloidin. Expression of TM1-TM2 chimera results in the assembly of stress fibers that are stained with TM antibody and phalloidin. The inset in the TM1-TM2 chimera cells (TM- panel) shows the presence of organized bundles of microfilaments at a higher magnification. The stress fibers in the chimera transfected cells costain with TM antibody and phalloidin and traverse the cell. The vector control cells lack TM-positive-microfilaments and resemble the parental DT cells. Both cell types are positive for GFP, as indicated. Bar 10μm.

Key Research accomplishments

- TM1 is downregulated in breast tumors, and the loss of TM1 may be a useful biomarker of breast cancer.
- Consistent downregulation of TM1 is a feature of breast cancer cells. Some lung and colon cancer cells, and melanoma cells express TM1.
- TM1 is a suppressor of MCF-7 and MDA MB 231 cells. This finding supports that TM1 is a general suppressor of cellular transformation.
- TM1 is downregulated by gene methylation and histone deacetylation.
- N-terminal modification of TM1 abolishes the tumor suppression
- Amino terminal portion of TM1 is critical for cytoskeletal reorganization and suppression of anchorage-independent growth
- TM2 is not a tumor suppressor.
- TM1 induces anoikis in breast cancer cells.
- The loss of TM1 may fecilatate the metastatic growth of breast tumor cells.

Reportable Outcomes

Manuscripts and Abstracts

1. Manuscripts:
   1.1. Vanya Shah, Bharadwaj, S. and **G, L. Prasad** (2001) *Oncogene* 20: 2112-2121.Cytoskeletal changes in tropomyosin-1 mediated reversion of phenotype Evidence for Rho kinase pathway.
   1.2. S. Bharadwaj and G. L. Prasad (2002) Tropomyosin-1 is downregulated by Promoter Methylation in Cancer Cells. Cancer Letters 183: 205-213.
   1.3. Suppression of transformed phenotype of breast cancer by tropomyosin-1 (2002). Kalyankar Mahadev, Gira Raval, Mark Willingham, Ethan M. Lange, Barbara Vonderhaar, David Salomon, and G. L. Prasad. Experimental Cell Research 279: 40-51. This paper has been selected by the Editor for presentation in the highlight section of the journal.
   1.4. Loss of expression of Tropomyosin-1, a Novel Class II Tumor Suppressor that induces anoikis, in Primary Breast Tumors (2003). Gira N. Raval[1], Shantaram Bharadwaj[1], Edward A. Levine, Mark C. Willingham, Randolph L. Geary, Tim Kute and G. L. Prasad. Oncogene 6194-6203. [1]Co-first authors
   1.5. Bharadwaj, S., Hitchcock-DeGregori, S., Thorburn, A., and **Prasad, G. L.** (2004). N Terminus Is Essential for Tropomyosin Functions: N-Terminal Modification Disrupts Stress Fiber Organization And Abolishes Anti-Oncogenic Effects Of Tropomyosin-1. *J. Biol. Chem.* 279, 14039-14048.
   1.6. Bharadwaj, S., Thanawala, R., Bon, G., Falcioni, R., and Prasad, G. L. Re-sensitization of breast cancer cells to anoikis by Tropomyosin-1 through cytoskeleton-dependent modulation of integrin activity and Rho kinase signaling *(manuscript submitted).*
   1.7. Bharadwaj, S., Shah, V., Tariq, F., Damartoski, B., Prasad, G. L. (2005) Amino terminal, but not the carboxy terminal, sequences of Tropomyosin-1 are essential for the induction of stress fiber assembly in neoplastic cells. *(manuscript submitted).*

2. **Abstracts**
   2.1. Tropomyosin-1, a novel tumor suppressor and a biomarker. Gira Raval, Shantaram Bharadwaj, Edward Levine1, Mark Willingham2, Randolph Geary1 and G. L. Prasad. Era of Hope, Army Breast Cancer Meeting. Orlando, FL, September 2002.
   2.2. Cytoskeletal Proteins as Regulators of Breast Cancer: Anoikis and Tumor Suppression by Tropomyosin-1, a Microfilament Stabilizing Protein. Shantaram Bharadwaj, Edward A Levine and G. L. Prasad. AACR Special Meeting Advances in Breast Cancer Research Genetics, Biology and Clinical Implications, October 8-12, 2003, Huntington Beach, CA.
   2.3. Cytoskeletal Proteins as inducers of Anoikis: Studies on Tropomyosin-1-mediated integrin activity and Rho kinase signaling. Shantaram Bharadwaj, Ruchi Thanawala and G. L. Prasad. Gordon Conference on Signaling and Adhesion Receptors (June 04, Bristol, RI).

Cell lines and reagents:

1. Adenoviral vectors that express TM1.
2. Chimeric Tropomyosins, and a variant TM1 molecules

3. MCF-7 and MDA MB 231 cells which express various isoforms of TM1, including TM1.
4. A TM1-specific antibody

Grants:

Several grant applications are now pending

Promotions:

The PI has been promoted to the rank of Associate Professor based on his funding from DOD.

The U.S. Army Medical Research and Materiel Command under DAMD-98-1-8162 supported this work.

Conclusions

In summary, the major accomplishments of our work for are two fold: First, we demonstrated, for the first time, that TM1 is downregulated in breast tumors. While TM1 has been known to bind and stabilize actin microfilaments for a long time, its role in modulating tumor growth is now becoming evident. Second, we have demonstrated that TM1 is a suppressor of the malignant growth of breast cancer. The discovery that TM1 expression is significantly downregulated in invasive breast tumors suggests TM1 could be used as a novel biomarker.

There is a significant increase in the detection of smaller breast masses due to sensitive mammographic techniques, and it is necessary to determine whether they are benign and have the potential to become malignant. A suitable molecular marker, such as TM1, which is altered only in malignant tissues may be a useful surrogate marker in this regard. Continued expression of TM1 at significant levels would indicate that the tissue is benign, or a decrease in TM1 expression would suggest a need for further evaluation.

TM1 may also serve as a marker to determine the endpoint or efficacy of novel therapies involving inhibition of DNA methyl transferases and histone deacetylases [11]. Since TM1 gene is silenced by gene methylation and histone deacetylation, these therapies would upregulate TM1 expression and other key genes that are essential for normal growth [11]. Upregulated TM1 may serve as a marker for the effectiveness of the drugs, and reactivated TM1 itself may contribute to the suppression of malignant growth, as shown in the culture experiments [2].

The finding that TM1 induces anoikis [3] may explain why TM1 is downregulated in primary breast tumors. The resistance to anoikis is considered to be a essential for metastatic growth [12]. Furthermore, we also find that TM1 inhibits binding of breast cancer cells to collagenI matrices specifically (work in progress). Binding to collagen is considered to be a necessary for efficient invasion of bone [13]. Therefore, investigations into how TM1 decreases binding to collagen may lead to better strategies to treat breast cancer. Work along those lines is in progress.

References

[1] B. Bhattacharya, G. L. Prasad, E. M. Valverius, D. S. Salomon, H. L. Cooper. Tropomyosins of human mammary epithelial cells: consistent defects of expression in mammary carcinoma cell lines, Cancer Research 50 (1990) 2105-2112.

[2] K. Mahadev, G. Raval, S. Bharadwaj, M. C. Willingham, E. M. Lange, B. K. V. Vonderhaar, D. Salomon, G. L. Prasad. Suppression of the transformed phenotype of breast cancer by tropomyosin-1, Experimental Cell Research. 279 (2002) 40-51.

[3] G. N. Raval, S. Bharadwaj, E. A. Levine, M. C. Willingham, R. L. Geary, T. Kute, G. L. Prasad. Loss of expression of tropomyosin-1, a novel class II tumor suppressor that induces anoikis, in primary breast tumors, Oncogene 22 (2003) 6194-6203.

[4] G. L. Prasad, R. A. Fuldner, H. L. Cooper. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene, Proc Natl Acad Sci U S A 90 (1993) 7039-7043.

[5] V. Shah, R. Braverman, G. L. Prasad. Suppression of Neoplastic Transformation and Regulation of Cytoskeleton by Tropomyosins, Somatic Cell Mol. Genetics 24 (1998) 273-280.

[6] R. H. Braverman, H. L. Cooper, H. S. Lee, G. L. Prasad. Anti-oncogenic effects of tropomyosin: isoform specificity and importance of protein coding sequences, Oncogene 13 (1996) 537-545.

[7] S. Bharadwaj, G. L. Prasad. Tropomyosin-1, a novel suppressor of cellular transformation is downregulated by promoter methylation in cancer cells, Cancer Letters 183 (2002) 205-213.

[8] S. Bharadwaj, R. Thanawala, G. Bon, R. Falcioni, G. L. Prasad. Re-sensitization of breast cancer cells to anoikis by Tropomyosin-1 through cytoskeleton-dependent modulation of integrin activity and Rho kinase signaling, (Manuscript communicated) (2005)

[9] S. Bharadwaj, S. Hitchcock-DeGregori, A. Thorburn, G. L. Prasad. N Terminus Is Essential for Tropomyosin Functions: N-Terminal Modification Disrupts Stress Fiber Organization And Abolishes Anti-Oncogenic Effects Of Tropomyosin-1, J. Biol. Chem. 279 (2004) 14039-14048.

[10] S. Bharadwaj, V. Shah, F. Tariq, B. Damartoski, G. L. Prasad. Amino terminal, but not the carboxy terminal, sequences of Tropomyosin-1 are essential for the induction of stress fiber assembly in neoplastic cells, Manuscript submitted. (2005)

[11] S. B. Baylin, M. Esteller, M. R. Rountree, K. E. Bachman, K. Schuebel, J. G. Herman. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer, Human Molecular Genetics 10 (2001) 687-692.

[12] S. M. Frisch, R. A. Screaton. Anoikis mechanisms, Curr Opin Cell Biol 13 (2001) 555-562.

[13] P. van der, H. Vloedgraven, S. Papapoulos, C. Lowick, W. Grzesik, J. Kerr, P. G. Robey. Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components, Lab Invest 77 (1997) 665-675.

List of Personnel Supported by this Award

1. G. L. Prasad, PI

Oncogene (2001) 20, 2112–2121

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APPENDIX 1

Cytoskeletal organization in tropomyosin-mediated reversion of *ras*-transformation: Evidence for Rho kinase pathway

Vanya Shah[3], Shantaram Bharadwaj[1,2], Kozo Kaibuchi[4] and GL Prasad*[,1,2]

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Tropomyosin (TM) family of cytoskeletal proteins is implicated in stabilizing actin microfilaments. Many TM isoforms, including tropomyosin-1 (TM1), are down-regulated in transformed cells. Previously we demonstrated that TM1 is a suppressor of the malignant transformation, and that TM1 reorganizes microfilaments in the transformed cells. To investigate how TM1 induces microfilament organization in transformed cells, we utilized *ras*-transformed NIH3T3 (DT) cells, and those transduced to express TM1, and/or TM2. Enhanced expression of TM1 alone, but not TM2, results in re-emergence of microfilaments; TM1, together with TM2 remarkably improves microfilament architecture. TM1 induced cytoskeletal reorganization involves an enhanced expression of caldesmon, but not vinculin, α-actinin, or gelsolin. In addition, TM1-induced cytoskeletal reorganization and the revertant phenotype appears to involve re-activation of RhoA controlled pathways in DT cells. RhoA expression, which is suppressed in DT cells, is significantly increased in TM1-expressing cells, without detectable changes in the expression of Rac or Cdc42. Furthermore, expression of a dominant negative Rho kinase, or treatment with Y-27632 disassembled microfilaments in normal NIH3T3 and in TM1 expressing cells. These data suggest that reactivation of Rho kinase directed pathways are critical for TM1-mediated microfilament assemblies. *Oncogene* (2001) **20**, 2112–2121.

Keywords: tropomyosin; cytoskeleton; Rho kinase

Introduction

Expression of many cytoskeletal proteins is down-regulated during neoplastic transformation, resulting in an altered cell morphology, reorganization of cytoarchitecture, cell motility, and possibly contributing to changes in gene expression by modulating intracellular signaling pathways (Button *et al.*, 1995; Janmey and Chaponnier, 1995). For example, many microfilament associated proteins such as α-actinin, vinculin, gelsolin and tropomyosins (TMs) are suppressed to varying degrees in many transformed cells (Ben-Ze'ev, 1997). Furthermore, restoration of these proteins inhibits the malignant phenotype of many different experimentally transformed cell lines, underscoring the pivotal role of cytoskeletal organization in maintaining a normal phenotype (Ayscough, 1998; Janmey and Chaponnier, 1995). Our laboratory has been interested in understanding the role of cytoskeletal proteins, in particular that of tropomyosins, in malignant transformation.

Tropomyosin (TM) family comprises of 5–7 different closely related isoforms, whose expression is altered in many transformed cells (Lin *et al.*, 1997; Pittenger *et al.*, 1994). For example, suppression of high M_r TMs, *viz.*, isoforms 1 and 2 (TM1 and TM2, respectively), is nearly universal in all the transformed cell lines tested, while elevated levels of the low M_r species such as TM4 and TM5 are reported in some malignant cell types (Bhattacharya *et al.*, 1990; Cooper *et al.*, 1985, 1987; Hendricks and Weintraub, 1981; Leavitt *et al.*, 1986; Matsumura *et al.*, 1983). Although all TMs bind to actin with varying binding affinities, the precise function of each of the isoforms remains largely unknown. Other actin binding proteins, such as caldesmon, also influence TM binding to actin to varying degrees (Pittenger *et al.*, 1995). Our efforts to elucidate the causal relationship between tropomyosin expression and cell phenotype have uncovered significant fundamental differences in the roles of TM isoforms in cell physiology (Braverman *et al.*, 1996; Prasad *et al.*, 1993; Shah *et al.*, 1998).

In DT (v-*ki-ras*-transformed NIH3T3) cells, TM1 expression is suppressed to 50% levels found in normal fibroblasts, but TM2 levels are essentially undetectable (Cooper *et al.*, 1985; Prasad *et al.*, 1993). DT cells are spindle shaped with no defined microfilament structures and are highly malignant. Transduction of DT cells with a cDNA encoding TM1 protein results in reorganization of cytoskeleton with well-defined microfilaments. More significantly, elevated TM1 levels inhibited the transformed phenotype of DT cells, culminating in a stable revertant phenotype (Braverman *et al.*, 1996; Prasad *et al.*, 1993). In addition, TM1 also suppresses v-*src*-induced transformed phenotype, suggesting that TM1 may be a general suppressor of transformation that belongs to class II tumor suppressors (Prasad *et al.*, 1999). On the contrary, restoration

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of expression of TM2 protein did not improve the cytoskeleton, and the cells remained transformed, resembling DT cells (Braverman *et al.*, 1996). Co-expression of TM2 with TM1, however, dramatically improved microfilaments, similar to those in normal NIH3T3 cells: DT cells expressing TM1 and TM2 exhibit a revertant phenotype (Shah *et al.*, 1998). It is intriguing that TM1, an actin binding protein reorganizes the cytoskeleton and restores the normal growth phenotype, while the other closely related TMs despite their ability to bind to actin are unable to modulate cytoarchitecture and growth phenotypes (Braverman *et al.*, 1996; Pittenger *et al.*, 1994). While TM1 alone is able to organize the other cytoskeletal proteins into microfilaments to an appreciable degree, together with TM2 it restores the cytoarchitecture to the extent found in normal cells.

The restored cytoskeleton may be important for the anti-oncogenic effects of TM1. In the transformed cells, along with TMs, the expression myosin light chain and many other key microfilament proteins are also suppressed (Ben-Ze'ev, 1997; Janmey and Chaponnier, 1995; Kumar and Chang, 1992). To elucidate the nature of TM1-induced formation of microfilaments, we have evaluated the changes in the expression of other key cytoskeletal proteins and tested the stability of TM1-induced cytoskeleton in the DT cell system described above. Reorganization of cytoskeleton, arising from the forced expression of TM1, will have to involve recruitment of some of the other cytoskeletal proteins from the existing pool; alternatively, through yet unknown mechanisms, TM1 might contribute to an increase in their synthesis. Our results, for the first time, show that TM1 reorganizes cytoskeleton via both of these mechanisms. We also provide evidence for the involvement of Rho-regulated pathways, in the emergence of microfilaments. These pathways could play a critical role in modulating gene expression, to culminate in a revertant phenotype.

Results

Expression of cytoskeletal proteins in TM1 mediated reversion

To investigate the role of TMs in cellular transformation, we generated several cell lines of DT expressing TM1 (DT/TM1 cells), TM2 (DT/TM2 cells) or both the TMs (DT/TM1-TM2 cells) by introduction of cDNAs encoding TM1, TM2 or both the TMs, respectively (Table 1; Figure 1) (Braverman *et al.*, 1996; Prasad *et al.*, 1991, 1993; Shah *et al.*, 1998). In transformed cells, in addition to TMs, many other proteins including those associated with microfilaments are also suppressed. For example, expression of gelsolin, vinculin, α-actinin and caldesmon is down-regulated in many transformed cells and tumors (Asch *et al.*, 1999; Ben-Ze'ev, 1997; Button *et al.*, 1995; Janmey and Chaponnier, 1995; Mielnicki *et al.*, 1999). Given the high degree of the malignant nature of DT cells, these changes are expected to be extensive. It should be noted that TM1 and TM2 share extensive homology, yet only TM1 is capable of reorganizing microfilaments and suppressing the Ras-induced transformed phenotype (Braverman *et al.*, 1996; Shah *et al.*, 1998). The properties of microfilaments induced by TM1 in DT cells could significantly differ because the expression of many components is likely to remain repressed due to the continued oncogenic action of Ras. To understand the molecular basis of reorganization of cytoskeleton in DT/TM1 and DT/TM1-TM2 cells, expression of some of the key microfilament-associated proteins was investigated in normal NIH3T3, DT, DT cells transduced with an empty pBNC vector (DT/V), DT/TM1, DT/TM2 and DT/TM1-TM2 cells.

Vinculin expression was significantly lower in DT and the derived cell lines compared to normal fibroblasts (Figure 2a), a finding consistent with the published data (Rodriguez Fernandez *et al.*, 1992). Immunoprecipitation followed by Western blotting with a specific antibody revealed that expression of vinculin remained low in the cell lines derived from DT, regardless of the phenotype. Then we investigated whether TM1 expression modulates distribution of vinculin in focal adhesions by confocal microscopy (Figure 2b). The samples were stained with anti-vinculin antibody optically, sectioned with a confocal microscope and the composite images are presented. In normal NIH3T3 cells, vinculin was well spread through out the cell body and organized as dense particles, indicative of focal adhesions. In DT cells, where a 25–30% reduction in vinculin levels is noted, a different organization, consistent with the lack of microfilaments, was observed: vinculin is mostly concentrated to the perinuclear area. In DT/TM1 cells, the vinculin staining is more spread out. In cells expressing both TM1 and TM2, however, vinculin was spread more evenly in the cell body, with the presence of 'speckled' appearance indicating a better organization. The samples were also stained for the presence of actin filaments by Texas-Red conjugated phalloidin (data not shown).

Caldesmon, which regulates binding of TMs to microfilaments, is also down regulated in transformed cells. Data of Figure 3a show that expression of caldesmon mRNA is suppressed 40% in DT, DT/V and DT/TM2 cells. However, in DT/TM1 (90%) and DT/TM1-TM2 (≈100%) cells caldesmon mRNA expression is nearly restored to the levels found in normal fibroblasts. Caldesmon protein levels were also enhanced in the revertant DT/TM1 and DT/TM1-TM2 cells, compared to the parental DT cells, as determined by immunoblotting (Figure 3b).

On the other hand, there are no consistent changes in the expression of α-actinin in NIH3T3, DT and the derived cell lines (data not shown). In normal fibroblasts, transformed and the revertant cells, α-actinin levels did not change appreciably. However, in cell lines expressing TM2, there appears to be a modest decrease in α-actinin levels. Western blotting with anti-

Table 1 DT cell model system

Cell line	*Derived from*	*Generated by*	*Growth phenotype*	*TM1 expression*	*TM2 expression*	*TM3 expression*
NIH3T3 cells	Parent cells	Parent cells	Normal	++ normal	++ normal	++ normal
DT	NIH3T3	Transformation with two copies of *v-Ki-ras*	Malignant	+ (50% decrease)	– (not detectable)	– (not detectable)
DT/TM1	DT	Transduction with TM1 cDNA	Revertant	⩾++ (enhanced)	– (not detectable)	– (not detectable)
DT/TM2	DT	Transfection with TM2 cDNA	Malignant	+ (same as in DT)	⩾++ (enhanced)	– (not detectable)
DT/TM1-TM2	DT/TM1	Transfection with TM2 cDNA	Revertant	⩾++ (enhanced)	⩾++ (enhanced)	– (not detectable)

Normal NIH3T3 cells have been transformed by two coies of *v-ki-ras* to generate DT cells, which express decreased TM1, and no detectable TM2 or TM3. Transduction of DT cells with a recombinant retrovirus to express high levels of TM1 reverts DT cells to normal phenotype with a well spread morphology and microfilaments (DT-TM1). Restoration of TM2, however, does not alter the cell morphology or the phenotype (DT-TM2). But co-expression of TM2 remarkably improves the microfilament architecture of DT/TM1 cells (DT/TM1-TM2). Growth phenotypes of the cell lines is indicated

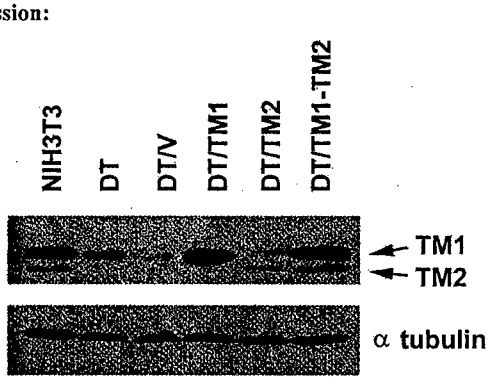

Figure 1 TM expression in DT cell lines: Western blotting of cell lysates probed with anti-TM antiserum that recognizes multiple TMs. Positions of TM1 and TM2 are indicated. Please note that TM2 and TM3 co-migrate in this experiment. Two dimensional gel analyses of TM expression from these cell lines are published previously (7, 13–16). Normal NIH3T3 cells express all the TM isoforms, while in DT cells (and in vector control DT/V), expression of TM2 and TM3 is undetectable, with a 50% decrease in TM1 expression. In DT/TM1 cells TM1 expression is enhanced, and DT/TM2 cells overexpress TM2. DT/TM1-TM2 cells manifest elevated levels of TM1 and TM2

gelsolin antibody revealed that there is a detectable decrease (in relation to tubulin expression) in the expression in DT and DT/V cells when compared to untransformed cells. Gelsolin levels did not significantly change in DT/TM1 or DT/TM2, but are enhanced in DT/TM1-TM2 cells, to the levels found in normal fibroblasts (data not shown).

Expression of Rho-family of regulatory proteins

Ras-related Rho family of GTPases is widely recognized as the regulators of cytoskeletal integrity and gene expression (Hall, 1998; Khosravi-Far *et al*., 1998). While Rho is implicated in the formation of microfilaments, Rac regulates lamellipodia and membrane

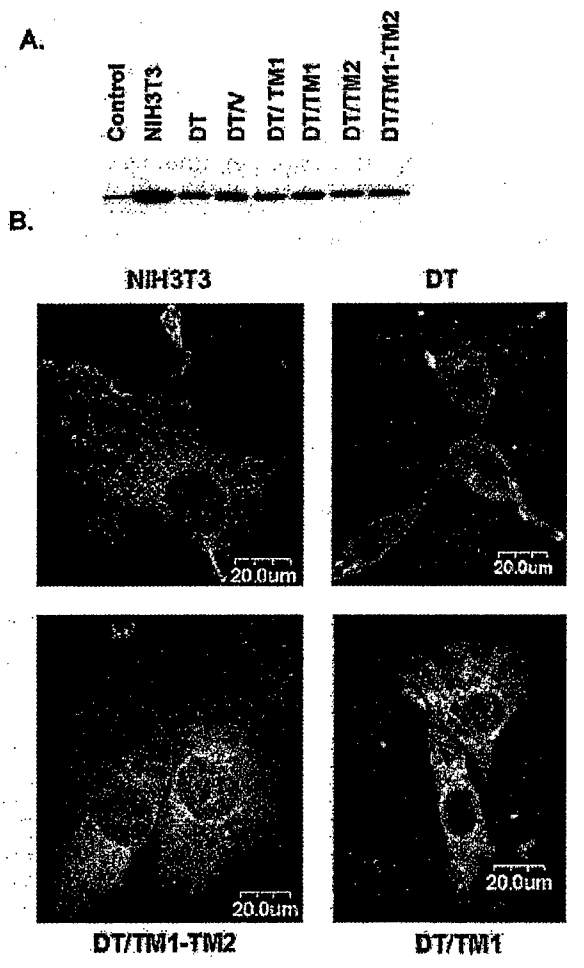

Figure 2 Vinculin expression: (**a**) Cytosolic extracts from the indicated cell lines were prepared, immunoprecipitated and Western blotted with anti-vinculin antibody, as described in Materials and methods. Control indicates a positive cell line-lysate supplied along with the antibody (Sigma chemical company). (**b**) Immunocytochemical staining of vinculin was performed with indicated cell lines. Samples were optically sectioned and the composite images were projected

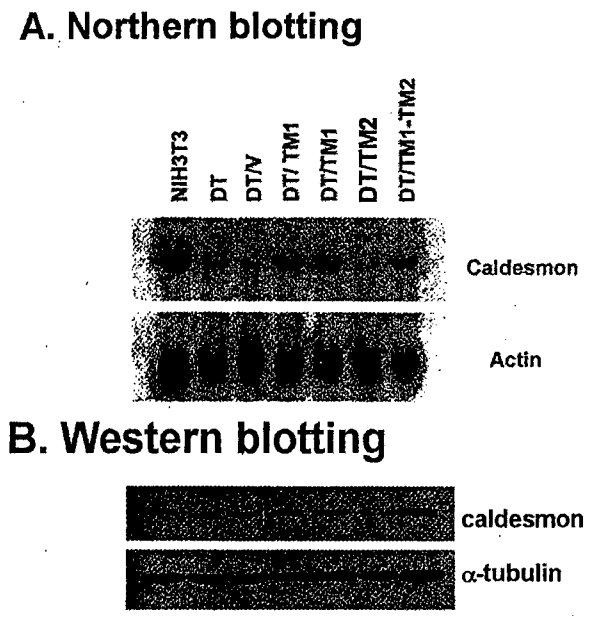

Figure 3 Caldesmon expression: (**a**) Northern blotting. 20 μg of total RNA from the indicated cell lines were loaded and hybridized against a full-length caldesmon cDNA. The membrane was stripped and reprobed with a β-actin probe for control purposes. (**b**) Western blotting. Total cytosolic extracts (100 μg) were reacted to anti-caldesmon antibody as described in Materials and methods. For control purposes, the membrane was stripped and probed with α-tubulin

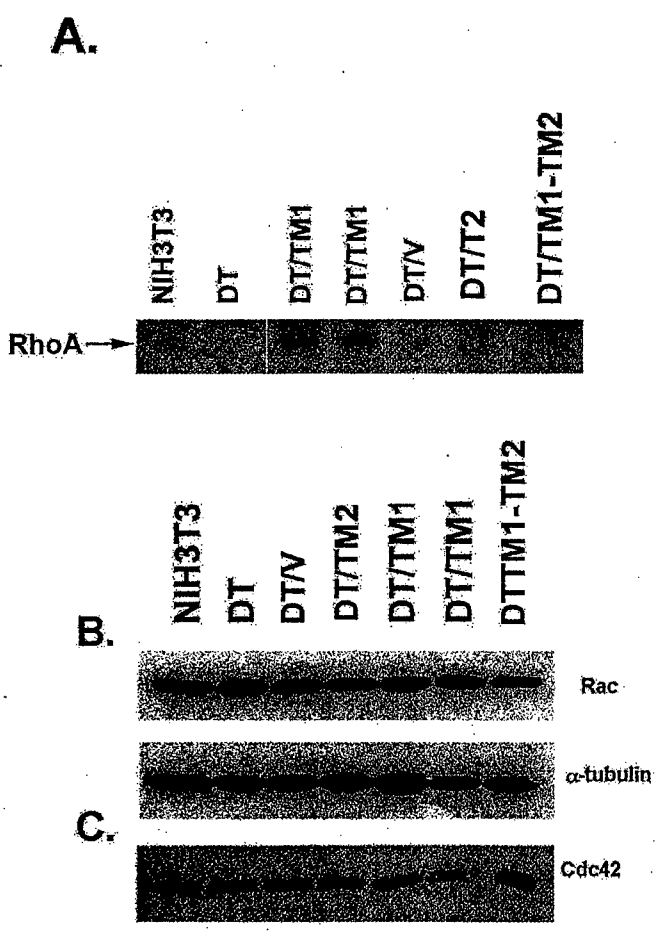

Figure 4 Expression of Rho family GTPases: (**a**) RhoA expression was determined by immunoprecipitations followed by Western blotting. Two hundred and fifty μg of total cytosolic proteins were immunoprecipitated with anti-RhoA antibodies. All the samples were immunoblotted for RhoA expression. (**b**) Rac expression was determined by immunoblotting using a monoclonal antibody (Upstate Biotechnologies) in the total cell lysates (25 μg). (**c**) Cdc42 expression was determined in 250 μg of total cytosolic proteins by immunoprecipitations and Western blotting using a monoclonal antibody (Santa Cruz Biotechnology)

ruffling and Cdc42 induces filopodia (microspikes). We investigated whether the observed reorganization and cell spreading involves changes in levels of Rho family of proteins. Immunoprecipitation followed by immunoblotting with anti-RhoA antibodies indicates that RhoA expression is lower in DT cells, while in normal fibroblasts it is readily detectable (Figure 4a). Similarly in the vector control DT cells and those expressing TM2, RhoA levels remained low. But in the DT/TM1 and DT/TM1-TM2 cells, RhoA expression is increased to normal levels. For control purposes and to test the specificity, the antibody was first reacted with the immunogen (blocking peptide) and used for immunoprecipitations (data not shown). In these experiments, as expected, no RhoA signal was detected in any of the lysates employed.

Western blotting with Rac antibody indicated a modest increase in DT cells, compared to NIH3T3 cells. But no clear-cut changes in Rac expression were discernible (Figure 4b). Immunoprecipitation and immunoblotting with anti-Cdc42 also did not reveal any appreciable changes (Figure 4c). These results indicate that TM1 expression enhances RhoA protein, which is likely to orchestrate the reorganization of cytoskeleton.

Stability of TM1 induced cytoskeleton

As noted above, the expression of many cytoskeletal proteins (including other TMs), is not completely restored in TM1-expressing DT cells. In addition, TMs are hypothesized to function as dimers; they could form homodimers as well as heterodimers. These and other interactions may not be completely restored in the revertants of DT, notwithstanding the re-emergence of microfilaments. Because of these reasons, we investigated whether TM1-induced cytoskeleton is as stable as the one in NIH3T3 cells: one established technique involves treatment of the cells with fungal toxins such as cytochalasins (Warren *et al.*, 1995). NIH3T3, DT/TM1 and DT/TM1-TM2 cells were incubated with cytochalasin D for different time periods and stained for TMs with an antibody (green) and actin (red). In normal NIH3T3 cells, after 5 min of addition of the drug, microfilaments were clearly visible and intact. After 10 min of treatment, although microfilaments were present, they appeared to be less prominent (Figure 5a). In contrast, in DT/TM1 cells, effects of cytochalasin D were profound; as early as in 5 min, the cells were rounded, with no evidence of any

organized actin filaments (Figure 5b). DT/TM1-TM2 cells were more resistant than TM1 alone expressing cells. Cytochalasin D treatment had modest, but readily detectable effects on actin microfilaments in 5 min, which were more pronounced in 10 min (Figure 5c). Accumulation of patches of actin along more defined structures is evident in 5 min of treatment, while more extensive destruction of cytoskeleton is observed in 10 min. Thus, restoration of TM1 expression is adequate to form microfilaments in DT cells, but these structures are rather labile. However, co-expression of TM2 not only significantly improves the cytoskeletal architecture, but also stabilizes microfilaments. In all of the cell lines, changes in actin staining closely mirrored that of TM. DT and DT/TM2 cells were not employed in this study, since they lack defined microfilaments to begin with.

Rho-kinase pathway

Rho-mediated contractility is critical for the assembly of stress fibers and focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). Rho kinase (p160ROCK) is a key effector of Rho-mediated signaling (Matsui *et al*., 1996; Narumiya *et al*., 1997). Among the pathways controlled by P160ROCK, regulation of microfilaments by phosphorylation of myosin light chain kinase and myosin-binding subunit of myosin phosphatase is a prominent one (Amano *et al*., 1996, 1997). Therefore, to investigate whether the re-emergence of microfilaments in TM1 expressing cells occurs through Rho kinase directed pathways, we employed two different approaches to disrupt possible p160ROCK signaling; transient transfection of DT/TM1 cells with dominant negative variants of

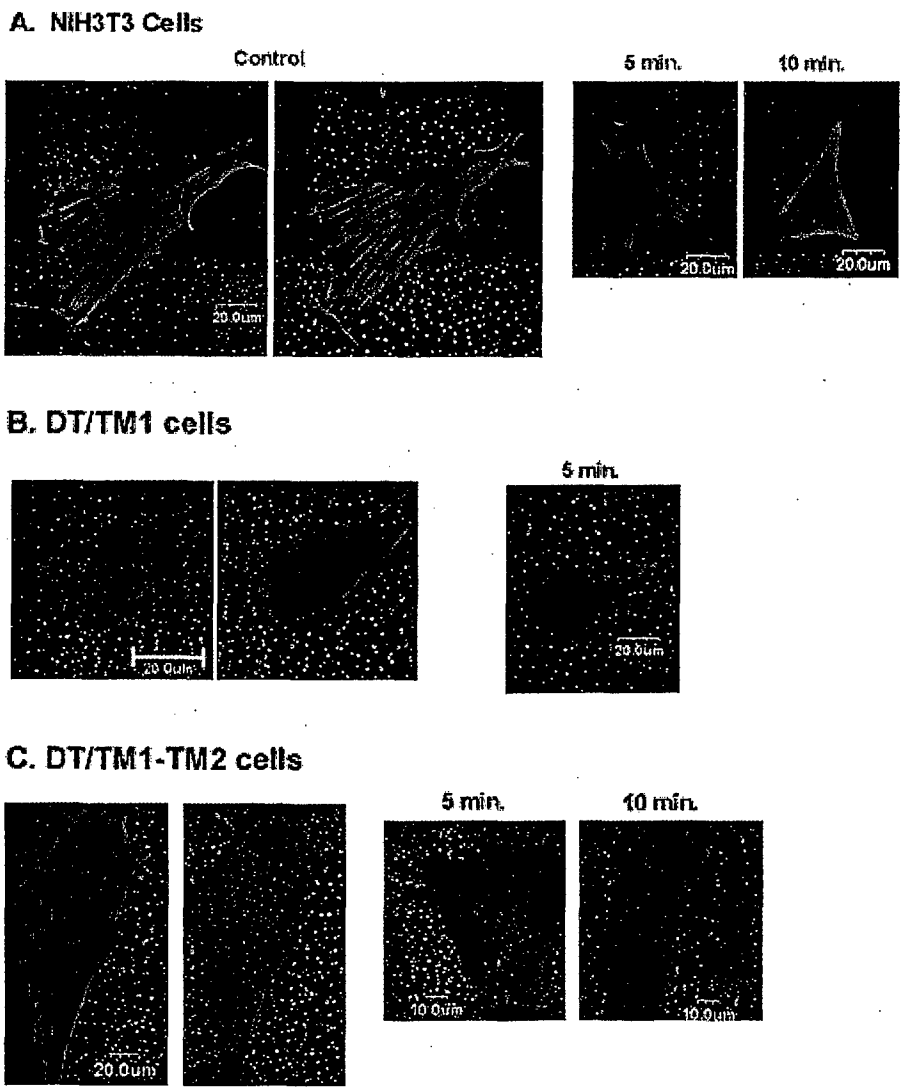

Figure 5 Stability of the cytoskeleton: NIH3T3 cells (a), DT/TM1 (b) and DT/TM1-TM2 (c) were treated with cytochalasin D at 0.5 μg/ml for 5 or 10 min and cells were stained for TMs and actin. Control samples were untreated. Green: TMs as visualized by anti-rabbit second antibody conjugated to FITC; and red: Actin microfilaments by Texas red conjugated phalloidin. DT/TM1 cells had essentially no cytoskeleton by 5 min of treatment and therefore, the 10 min treatment data are not shown. Since the actin-staining and TM staining was essentially identical, only actin pattern was shown for the treatments

p160Rock (Amano *et al.*, 1997), and treatment of cells with a specific inhibitor of p160ROCK, Y27632 were used (Sahai *et al.*, 1999).

We tested the importance of Rho kinase signaling in maintenance of microfilaments by transient transfection of DT/TM1-TM2 cells with a dominant negative Rho kinase construct, CAT-KD (Amano *et al.*, 1997; Chihara *et al.*, 1997). NIH3T3 cells were also transfected with CAT-KD for control purposes. In this experiment, actin filaments were visualized by staining with phalloidin: simultaneous staining for *myc* tag allowed the detection of transfected cells (Figure 6a,c), because the construct was tagged with a myc epitope. As evident from Figure 6b,d, expression of CAT-KD resulted in a severe disruption of microfilaments in NIH3T3 cells and DT/TM-TM2 cells. CAT-KD expression resulted in aggregation of actin into patches, with a profound decrease in the stress fibers. Similar results were obtained with DT/TM1 cells also (data not shown).

Treatment of NIH3T3 cells with 20 μM Y27632 for 15 min did not have significant effects on the cell spreading, but resulted in a decrease of microfilaments as observed by actin staining (Figure 7). A significant loss of microfilaments and shrinkage of cell mass was, however, evident by 30 min. Tropomyosin staining pattern was identical to that of f-actin in all the cell types also, and hence data are not presented. In DT cells, where no detectable microfilaments are absent, the effects of the drug were less obvious. Incubation with Y27632 for 15 and 30 min resulted in a similar morphology (Figure 7c,d). The spindle shaped DT cells assumed a round morphology with cytoplasmic extensions containing actin and TMs. In DT/TM1 and DT/TM1-TM2 cells, the effects of the inhibitor were far more extensive. Fifteen minutes of treatment of the drug resulted in severe disruption of microfilament network and cell morphology of the revertants (Figure 7e,g), although the effects were more pronounced in DT/TM1 cells (Figure 7e) than in DT/

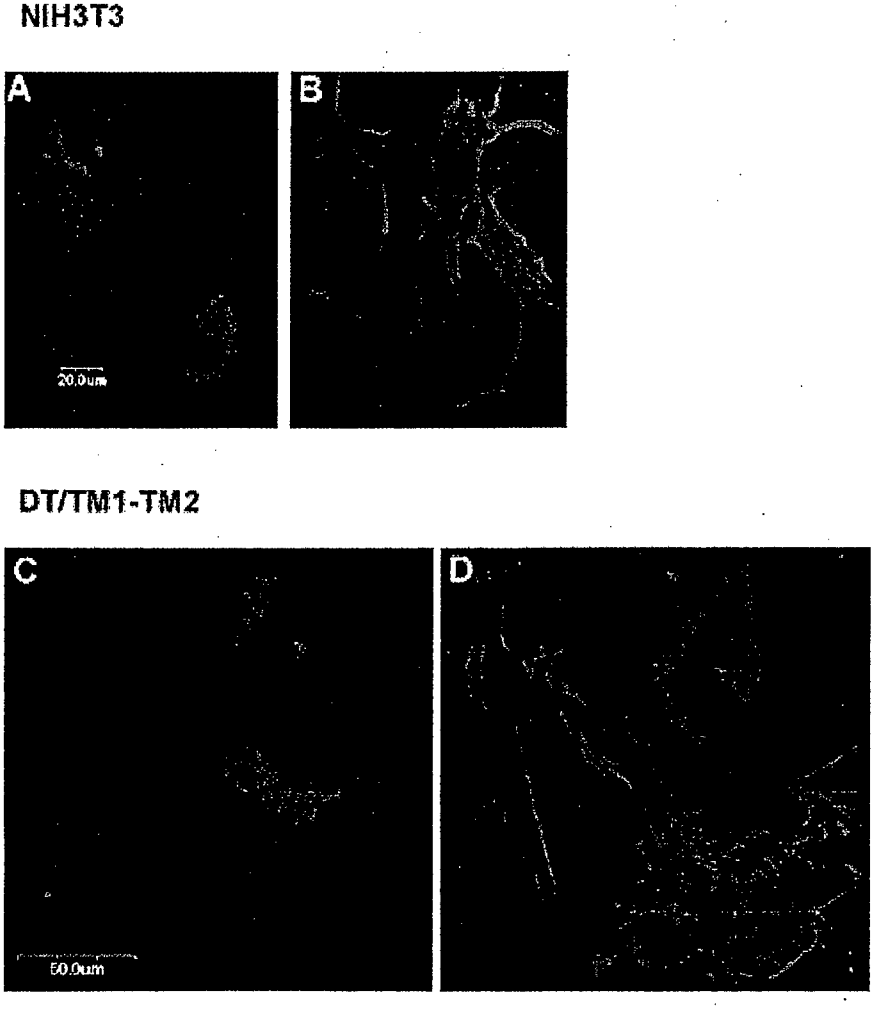

Figure 6 Effect of expression of dominant negative Rho kinase expression: Myc-epitope tagged dominant negative Rho kinase constructs (pEF-BOS-*myc* Rho kinase/CAT-KD) was used for transfection of NIH3T3 (**a,b**) and DT/TM1-TM2 (**c,d**) cells. Cells were stained for the expression of the transfected cDNA using anti-*myc*-epitope antibody (green), and microfilaments by Texas Red conjugated phalloidin. Images were viewed with an Olympus confocal microscope. Microfilament organization was severely disrupted in the transfected cells, as evidenced by patchy staining of actin, in the cells expressing CAT-KD (green)

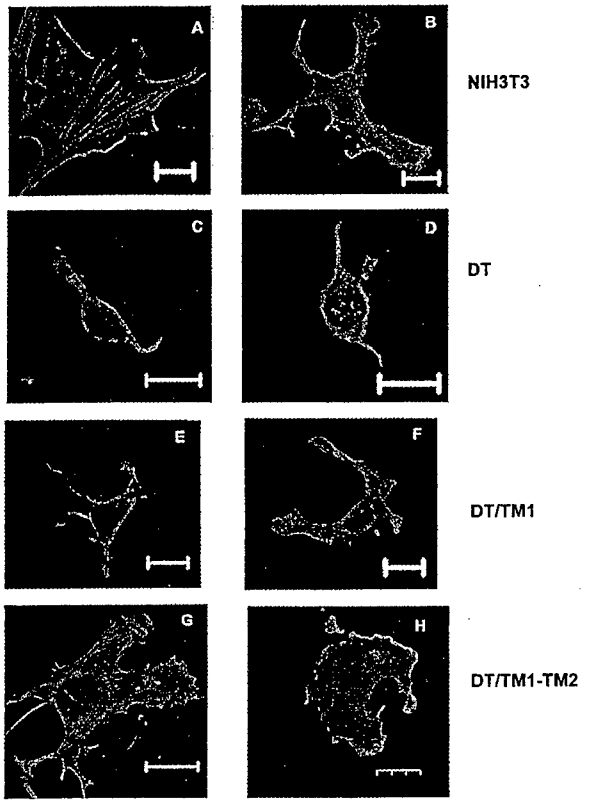

Figure 7 Effect of Y-27632 (Rho kinase inhibitor) on TM1-induced cytoskeleton: NIH3T3 (**a,b**), DT (**c,d**), DT/TM1 (**e,f**) and DT/TM1-TM2 (**g,h**) cells were treated with 20 μM of Y27632 for 15 min (**a,c,e,g**), or for 30 min (**b,d,f,h**). Cells were stained with phalloidin and photographed using a confocal microscope. The sizing bar indicates 20 μm

TM1-TM2 cells (Figure 7g). A lack of any organized microfilaments was evident in both cell types (Figure 7e–h). It should be noted that in NIH3T3 cells treated with Y-27632 a few microfilaments still remain (Figure 7b), indicating either a partial inhibition of the enzyme, or a possible participation of a different pathway. Under these conditions, however, in DT/TM1 and DT/TM1-TM2 cells complete disintegration of microfilament network is evident (Figure 7f,h). These data indicate that p160ROCK signaling is critical in the maintenance of microfilaments induced by TM1.

Discussion

Decreased expression of many actin-binding proteins, including that of TM1, in transformed cells is considered to be a contributing factor of the neoplastic phenotype (Ben-Ze'ev, 1997; Button *et al*., 1995; Janmey and Chaponnier, 1995). Restoration of TM1 expression abolishes the malignant phenotype, indicating that TM1 is a class II tumor suppressor (Prasad *et al*., 1999). Nevertheless, the underlying pathways involved in cytoskeletal reorganization by TM1 in the transformed cells are not completely understood.

We hypothesized that TM1-induced reversion of the malignant phenotype involves the restructuring of microfilaments and the modulation of signaling pathways controlled by *ras* oncogene. Reorganization of microfilaments requires participation of many other cytoskeletal proteins. In transformed cells, the levels of other actin-binding proteins, in addition to tropomyosins, are suppressed. In DT/TM1, DT/TM2 and DT/TM1-TM2 cells, expression of TM1, TM2 and both TMs is restored respectively, but the levels of other cytoskeletal proteins are not experimentally altered. In fact, expression of α-actinin, gelsolin and vinculin which are also known suppressors of transformation (Gluck *et al*., 1993; Rodriguez Fernandez *et al*., 1992) are not consistently altered in our revertant cells (data not shown). However, vinculin appeared to organize better in the revertant cells, perhaps reflecting improved microfilament architecture (Figure 2b). These data are consistent with the earlier reports that TM expression is not altered in the revertants produced by forced expression of either α-actinin or vinculin, thus suggesting the possible modes of reversion of the transformed phenotype by these cytoskeletal proteins could be distinct (Gluck *et al*., 1993; Rodriguez Fernandez *et al*., 1992).

A more notable and significant change is that caldesmon expression is specifically restored in TM1-expressing cells, but not in TM2 expressing transformed DT/TM2 cells, thus pointing to interactions between TM1 and caldesmon (Figure 3). Several lines of evidence suggest that caldesmon, which undergoes mitosis-specific phosphorylation and binds to actin in Ca^{2+}/calmodulin-regulated fashion, functions synergistically with TMs to modulate the integrity of actin cytoskeleton (Huber, 1997; Matsumura and Yamashiro, 1993); first, TMs and caldesmon together inhibit the gel severing activity of gelsolin (Ishikawa *et al*., 1989a,b); second, TMs and caldesmon enhance each others binding to actin (Ishikawa *et al*., 1998; Novy *et al*., 1993); third, caldesmon preferentially increases TM1-binding to actin when compared with that of other TM isoforms with actin (Pittenger *et al*., 1995); fourth, fascin binding with actin is shown to be completely inhibited by caldesmon and TMs together, but little or no effects were observed with either protein alone (Ishikawa *et al*., 1998); and, fifth, caldesmon when phosphorylated, or bound by calmodulin, dissociates from actin. This results in weakening of TM-actin association, exposing actin filaments to the action of gel-severing proteins (Pittenger *et al*., 1995). Furthermore, increased expression of caldesmon stabilizes microfilaments (Warren *et al*., 1995, 1996), and caldesmon levels are decreased in many transformed cells, as shown in Figure 3, and by others (Button *et al*., 1995; Novy *et al*., 1991). More recent studies implicate caldesmon in the regulation of actomyosin contractility and adhesion-dependent signaling in fibroblasts (Helfman *et al*., 1999).

TM1-induced cytoskeleton in DT cells appears to be less stable compared to that in NIH3T3 cells. The entry of several other proteins into the cytoskeletal

compartment may also remain impaired in DT/TM1 cells, due to the continued activity of *ras* oncogene in these cells. However, co-expression of TM2 resulted in a marked improvement both in the organization and stability of cytoskeleton, as judged by the sensitivity to cytochalasin D. It is known that heterodimers of TMs are thermodynamically preferred over homodimers (Jancso and Graceffa, 1991). Our previous studies showed that in DT/TM1 cells, homodimers of TM1 are formed initially (Prasad *et al.*, 1994). They are presumed to exchange with heterodimers consisting of TM1 and possibly with other low M_r TMs, since TM2 and TM3 are nearly lacking in these cells (Prasad *et al.*, 1994). In DT/TM1-TM2 cells, where both TM1 and TM2 are available, heterodimers of TM1 and TM2 are likely to exist, in addition to homodimers of TM1 and homodimers of TM2 (Shah *et al.*, 1998). These, dynamic multiple TM associations may exert more stabilizing influence and protect microfilaments.

A possible pathway through which TM1 could induce microfilaments and affect the cell phenotype is via Rho directed signaling (Narumiya *et al.*, 1997). Three different lines of evidence support the involvement of Rho proteins in the re-emergence of microfilaments: (1) RhoA expression is enhanced in TM1 expressing cells, but not in the cell lines of DT or DT/TM2 that exhibit transformed phenotype and lack microfilaments; such changes, however, were not detected in the levels of Rac1 and Cdc42; (2) inhibition of p160ROCK with specific inhibitor, Y27632, lead to the dissolution of microfilaments in normal NIH3T3 cells and TM1 expressing derivatives of DT cells; and (3) expression of a dominant negative Rho kinase, CAT-KD, interfered with the microfilament organization in NIH3T3 and DT/TM1-TM2 cells. The demonstration that RhoA is involved in TM1-induced microfilament formation complements the earlier finding that RhoA pathway may be inactivated in Ras transformation (Izawa *et al.*, 1998).

Our results are also consistent with previous data utilizing dominant active variants Rho and Rho-kinase that Rho proteins are required for the assembly of stress fibers (Izawa *et al.*, 1998). However, our results demonstrating the involvement of Rho proteins in restoration of microfilaments differ with those of other researchers who showed that Rho proteins are required for Ras transformation, and constitutively activated of RhoA could transform the cells (Khosravi-Far *et al.*, 1995; Prendergast *et al.*, 1995; Qiu *et al.*, 1995). Enhanced TM1 expression resulted in elevated RhoA proteins, and microfilaments appears to be assembled via p160ROCK pathway. Thus, in DT cells, oncogenic Ras signaling may be channeled via Rac and Cdc42 to manifest the full malignant phenotype to compensate for decreased Rho, as shown in other systems (Izawa *et al.*, 1998; Moorman *et al.*, 1999). Another possible explanation is that DT cells are very potently transformed, and the effects of oncogenic Ras signaling could be more intense. For example, DT cells form foci with $\geqslant 80\%$ efficiency in anchorage independence assays, and with inoculation of as few as 1000 cells tumor growth can be observed in 7–10 days in tumorigenesis assays (Prasad *et al.*, 1993). Notwithstanding the highly malignant nature of DT cells, restoration of TM1 expression reverts DT cells. Whether reconstituted RhoA protein is also responsible for inhibition of other downstream signaling of oncogenic Ras in this system is unclear at present, and is under investigation.

Our data indicate that p160ROCK is essential in the assembly of stress fibers in TM1-induced cytoskeletal reorganization, indicating the similarity between the pathways operating in the revertants and in normal cells. These are likely to include RhoA directed activation of Rho kinase, which in turn stimulates contractility to assemble microfilaments (Amano *et al.*, 1996; Chrzanowska-Wodnicka and Burridge, 1996). For example, Y-27632 was shown to disrupt microfilament network in NIH3T3 cells (Sahai *et al.*, 1999). In addition, transformation and cytoskeletal changes induced by constitutively active RhoA are inhibited by Y27632. The data presented herein suggest that upregulation and activation of the endogenous RhoA is likely to mediate TM1-induced cytoskeletal reorganization. The dominant negative variants of Rho kinase and Y-27632, although appear to elicit different morphological effects, are able to disrupt the stress fibers in the revertants employed in this study.

We propose that TM1-induced stress fiber assembly occurs via the following model. TM1 induces restoration of RhoA and caldesmon, reorganizes vinculin at the focal adhesions in DT cells. It is likely that the expression of caldesmon could be elevated in the revertant cells under the influence of RhoA. Caldesmon expression is shown to be regulated by SRF (Momiyama *et al.*, 1998), which in turn is regulated by RhoA (Montaner *et al.*, 1999; Spencer and Misra, 1999; Treisman *et al.*, 1998; Zohar *et al.*, 1998). Enhanced RhoA levels reactivate p160ROCK pathway, which is essential for maintenance of microfilaments. It is recently demonstrated that multiple factors, including cytoskeletal structures regulate Rho activity (Ren *et al.*, 1999). Co-expression of TM1 and TM2 significantly improves microfilament architecture and stability. Whether the restored cytoskeleton is essential in establishing and maintaining a revertant phenotype induced by TM1 is under investigation.

Materials and methods

Cell culture

Culture conditions have been described for all cell lines used in this study (Braverman *et al.*, 1996). Briefly, NIH3T3, DT cells and the derivative cell lines are cultured in DMEM high glucose medium (Life Technologies, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) in the presence of L-glutamine and penicillin and streptomycin. Cell lines derived from DT were supplemented with appropriate drugs, depending on the selection marker. The medium for DT/TM1 cells contained 200 μg/ml of Geneticin (Life Technologies), while xanthine (250 μg/ml; Sigma Chemical Company, St. Louis, MO, USA) and mycophenolic acid (2.5 mg/ml; Sigma) are present in the

culture medium for DT/TM2 and DT/TM1-TM2 cells; in addition, DT/TM1-TM2 cell media also contained G418.

Cytochalasin D (Sigma) was added to medium at (0.5 μg/ml) to test the stability of microfilaments for indicated times and the cells were processed for immunofluorescence experiments, as described below. Rho kinase inhibitor, Y-27632 was generously provided by Yoshitomi Pharmaceutical Industries, Ltd., Japan, and was added to the medium at 20 μM.

Western blotting and immunoprecipitations

Cell lysates were prepared as previously described in 1% NP-40/0.25% deoxycholate containing 50 mM Tris buffer pH 7.4 and 0.15 M NaCl, with protease inhibitors (Shah *et al.*, 1998). 100 μg of protein was subjected to electrophoresis on SDS-polyacrylamide gels and electrophoretically transferred to Nytran (S and S, Keene, NH, USA). Western blotting was performed using antibodies against caldesmon, vinculin, gelsolin, α-actinin, Rac, CDC42 and RhoA proteins with appropriate second antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and Renaissance chemiluminescence kit (NEN, Boston, MA, USA). The blots were routinely reprobed upon treatment with 2% SDS and 5% 2-mercaptoethanol at 55°C with an antibody against α-tubulin for load control. Immunoprecipitations of cell lysates (250–500 μg of protein) were performed with relevant primary antibodies, and the same primary antibodies were used for detection in Western blotting.

Immunofluorescence

Cells were cultured in Nunc chamber slides, and treated with either cytochalasin D or Rho kinase inhibitor as needed. Cells were gently washed with phosphate buffered saline, fixed in 3.7% paraformaldehyde and extracted in 0.5% Triton X-100 for 15 min. The samples were incubated with the primary antibodies, followed by appropriate FITC-conjugated second antibodies (Molecular Probes, Eugene, OR, USA). When necessary the samples were also stained with phalloidin conjugated with Texas Red (Molecular Probes) and mounted in Prolong Antifade (Molecular Probes, Eugene, OR, USA) according to the manufacturer. The samples were viewed with an Olympus laser scanning confocal microscope, or a Zeiss LSM510 confocal microscope (Shah *et al.*, 1998).

Northern blotting

Total RNA was extracted from cultured cells and probed with a caldesmon cDNA probe. The same blot was stripped and reprobed with βactin cDNA as described earlier to normalize for RNA loading (Prasad *et al.*, 1993).

Plasmids and transfections

A dominant negative form of Rho kinase, CAT-KD was previously described (Amano *et al.*, 1996; 1997; Chihara *et al.*, 1997). pEF-Bos-*myc*-Rhokinase CAT-KD plasmid was used for transfection of normal NIH3T3 and DT/TM1-TM2 cells using DOTAP (Roche Molecular Biochemicals, Indianapolis, IN, USA), according to the manufacturer's guidelines. Forty eight hours after transfection, the cells were fixed and processed for immunocytochemistry as described above.

Acknowledgments

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References

Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y and Kaibuchi K. (1997). *Science*, **275**, 1308–1311.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y and Kaibuchi K. (1996). *J. Biol. Chem.*, **271**, 20246–20249.

Asch HL, Winston JS, Edge SB, Stomper PC and Asch BB. (1999). *Breast Cancer Research and Treatment*, **55**, 179–188.

Ayscough KR. (1998). *Curr. Opin. Cell Biol.*, **10**, 102–111.

Ben-Ze'ev A. (1997). *Curr. Opin. Cell Biol.*, **9**, 99–108.

Bhattacharya B, Prasad GL, Valverius EM, Salomon DS and Cooper HL. (1990). *Cancer Res.*, **50**, 2105–2112.

Braverman RH, Cooper HL, Lee HS and Prasad GL. (1996). *Oncogene*, **13**, 537–545.

Button E, Shapland C and Lawson D. (1995). *Cell Motil. Cytoskeleton*, **30**, 247–251.

Chihara K, Amano M, Nakamura N, Yano T, Shibata M, Tokui T, Ichikawa H, Ikebe R, Ikebe M and Kaibuchi K. (1997). *J. Biol. Chem.*, **272**, 25121–25127.

Chrzanowska-Wodnicka M and Burridge K. (1996). *J. Cell Biol.*, **133**, 1403–1415.

Cooper HL, Bhattacharya B, Bassin RH and Salomon DS. (1987). *Cancer Res.*, **47**, 4493–4500.

Cooper HL, Feuerstein N, Noda M and Bassin RH. (1985). *Mol. Cell Biol.*, **5**, 972–983.

Gluck U, Kwiatkowski DJ and Ben-Ze'ev A. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 383–387.

Hall A. (1998). *Science*, **279**, 509–514.

Helfman DM, Levy ET, Berthier C, Shtutman M, Riveline D, Grosheva I, Lachish-Zalait A, Elbaum M and Bershadsky AD. (1999). *Mol. Biol. Cell*, **10**, 3097–3112.

Hendricks M and Weintraub H. (1981). *Proc. Natl. Acad. Sci. USA*, **78**, 5633–5637.

Huber PA. (1997). *Int. J. Biochem. Cell Biol.*, **29**, 1047–1051.

Ishikawa R, Yamashiro S, Kohama K and Matsumura F. (1998). *J. Biol. Chem.*, **273**, 26991–26997.

Ishikawa R, Yamashiro S and Matsumura F. (1989a). *J. Biol. Chem.*, **264**, 16764–16770.

Ishikawa R, Yamashiro S and Matsumura F. (1989b). *J. Biol. Chem.*, **264**, 7490–7497.

Izawa I, Amano M, Chihara K, Yamamoto T and Kaibuchi K. (1998). *Oncogene*, **17**, 2863–2871.

Jancso A and Graceffa P. (1991). *J. Biol. Chem.*, **266**, 5891–5897.
Janmey PA and Chaponnier C. (1995). *Curr. Opin. Cell Biol.*, **7**, 111–117.
Joneson T, White MA, Wigler MH and Bar-Sagi D. (1996). *Science*, **271**, 810–812.
Khosravi-Far R, Campbell S, Rossman KL and Der CJ. (1998). *Adv. Cancer Res.*, **72**, 57–107.
Khosravi-Far R, Solski PA, Clark GJ, Kinch MS and Der CJ. (1995). *Mol. Cell. Biol.*, **15**, 6443–6453.
Kumar CC and Chang C. (1992). *Cell Growth Differ.*, **3**, 1–10.
Leavitt J, Latter G, Lutomski L, Goldstein D and Burbeck S. (1986). *Mol. Cell Biol.*, **6**, 2721–2726.
Lin JJ, Warren KS, Wamboldt DD, Wang T and Lin JL. (1997). *International Review of Cytology*, **170**, 1–38.
Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A and Kaibuchi K. (1996). *EMBO J.*, **15**, 2208–2216.
Matsumura F, Lin JJ, Yamashiro-Matsumura S, Thomas GP and Topp WC. (1983). *J. Biol. Chem.*, **258**, 13954–13964.
Matsumura F and Yamashiro S. (1993). *Curr. Opin. Cell Biol.*, **5**, 70–76.
Mielnicki LM, Ying AM, Head KL, Asch HL and Asch BB. (1999). *Exp. Cell Res.*, **249**, 161–176.
Momiyama T, Hayashi K, Obata H, Chimori Y, Nishida T, Ito T, Kamiike W, Matsuda H and Sobue K. (1998). *Biochem. Biophys. Res. Comm.*, **242**, 429–435.
Montaner S, Perona R, Saniger L and Lacal JC. (1999). *J. Biol. Chem.*, **274**, 8506–8515.
Moorman JP, Luu D, Wickham J, Bobak DA and Hahn CS. (1999). *Oncogene*, **18**, 47–57.
Narumiya S, Ishizaki T and Watanabe N. (1997). *FEBS Lett.*, **410**, 68–72.
Novy RE, Lin JL and Lin JJ. (1991). *J. Biol. Chem.*, **266**, 16917–16924.
Novy RE, Sellers JR, Liu LF and Lin JJ. (1993). *Cell Motil. Cytoskeleton*, **26**, 248–261.
Pittenger MF, Kazzaz JA and Helfman DM. (1994). *Curr. Opin. Cell Biol.*, **6**, 96–104.
Pittenger MF, Kistler A and Helfman DM. (1995). *J. Cell Sci.*, **108**, 3253–3265.
Prasad GL, Fuldner RA and Cooper HL. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 7039–7043.
Prasad GL, Fuldner RA, Braverman R, McDuffie E and Cooper HL. (1994). *Eur. J. Biol. Chem.*, **224**, 1–10.
Prasad GL, Masuelli L, Raj MH and Harindranath N. (1999). *Oncogene*, **18**, 2027–2031.
Prasad GL, Meissner PS, Sheer D and Cooper HL. (1991). *Biochem. Biophys. Res. Comm.*, **177**, 1068–1075.
Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF and Der CJ. (1995). *Oncogene*, **10**, 2289–2296.
Qiu RG, Chen J, McCormick F and Symons M. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 11781–11785.
Ren XD, Kiosses WB and Schwartz MA. (1999). *EMBO J.*, **18**, 578–585.
Rodriguez Fernandez JL, Geiger B, Salomon D, Sabanay I, Zoller M and Ben-Ze'ev A. (1992). *J. Cell. Biol.*, **119**, 427–438.
Sahai E, Ishizaki T, Narumiya S and Treisman R. (1999). *Current Biology*, **9**, 136–145.
Shah V, Braverman R and Prasad GL. (1998). *Somatic Cell. Mol. Gen.*, **24**, 273–280.
Spencer JA and Misra RP. (1999). *Oncogene*, **18**, 7319–7327.
Treisman R, Alberts AS and Sahai E. (1998). *Cold Spring Harbor Symposia on Quantitative Biology*, **63**, 643–651.
Warren KS, Lin JL, McDermott JP and Lin JJ. (1995). *J. Cell. Biol.*, **129**, 697–708.
Warren KS, Shutt DC, McDermott JP, Lin JL, Soll DR and Lin JJ. (1996). *Cell. Motil. Cytoskeleton*, **34**, 215–229.
Zohar M, Teramoto H, Katz BZ, Yamada KM and Gutkind JS. (1998). *Oncogene*, **17**, 991–998.

APPENDIX 2

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Tropomyosin-1, a novel suppressor of cellular transformation is downregulated by promoter methylation in cancer cells

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Abstract

Tropomyosins (TMs) are a family of microfilament binding proteins, which are suppressed in the transformed cells. We have investigated the mechanism of suppression of TMs, in particular that of tropomyosin-1 (TM1), in breast cancer cells. Inhibition of DNA methyl transferase with 5-aza-2′-deoxycytidine (AZA) alone did not induce TM1 expression. However, combined treatment of trichostatin A (TSA) and AZA resulted in readily detectable expression of TM1, but not that of other TM isoforms. Upregulation of TM1 expression paralleled with the reemergence of TM1 containing microfilaments, and in abolition of anchorage-independent growth. The synergistic action of AZA and TSA in reactivation of TM1 gene was also evident in ras-transformed fibroblasts. These data, for the first time, show that hypermethylation of TM1 gene and chromatin remodeling are the predominant mechanisms by which TM1 expression is downregulated in breast cancer cells.

Keywords: Tropomyosin; Methylation; Histone acetylation

1. Introduction

Tropomyosins (TMs) are important actin binding proteins that stabilize microfilaments from the action of gel severing proteins [1,2]. Non-muscle cells elaborate multiple isoforms of TMs, some of which are expressed with a high degree of tissue specificity by alternate splicing. TMs are generally grouped into high (284 amino acids) and low (248 amino acids) M_r species, and they share significant sequence homology among the individual isoforms and across the species. Nevertheless, TMs exhibit substantial diversity in terms of their size, binding affinity to actin, subcellular localization and in interactions with other proteins, thus suggesting that individual TMs may differ in their biological functions. For example, TM1, a high M_r TM isoform with 284 amino acids, is expressed in fibroblasts and epithelial cells, but not in skeletal muscle tissue. TM1 has been shown to bind to actin with a higher affinity, and its interactions with actin are preferentially promoted by other TM isoforms, or other actin binding proteins such as caldesmon.

The expression of high M_r TMs is downregulated in transformed cells [1,2]. Two reports indicate that downregulation of TMs is rapid and precedes the morphological transformation of growth factor induced transformation [3,4]. Studies on the mechanism of downregulation of tropomyosins in fibroblasts suggested that both MEK-dependent and -independent signaling pathways are involved [5,6].

Previous work from this laboratory has shown that

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in breast cancer cells, multiple TM isoforms are suppressed [7]. Additionally, the expression of TM1 was consistently lost in breast cancer cells, indicating that loss of TM1 expression could be a common biochemical event in mammary carcinogenesis. Further investigations revealed that restoration of TM1 suppresses the malignant phenotype of oncogene-transformed cells and breast cancer cells, suggesting that TM1 is a general suppressor of neoplastic growth phenotype [8–10]. The focus of the present work is to elucidate the mechanism of downregulation of TM1 in breast cancer cells. Several important genes involved in a wide range of pivotal cellular processes are transcriptionally silenced by gene methylation in breast cancer [11]. Therefore, we examined the role of promoter methylation of TM1 gene as a possible mechanism in the loss of TM1 expression in cancer cells.

2. Materials and methods

Cell culture and drug treatments: MCF-7 and MDA MB231 cells were purchased from ATCC, and the sources and culture conditions of MCF10A and DT cells have been previously described [7,12]. 5-Aza-2′-deoxycytidine (AZA) was added to the subconfluent cultures at a final concentration of 8.8 μM. For the experiments involving combined treatment of AZA and TSA, TSA was added to the culture medium at 300 nM after pretreating the cells with AZA for 24 h. The cells were routinely harvested as indicated.

RNA isolation and northern blotting for the expression of TMs was performed as described elsewhere [12]. For detection of TM1, a full length cDNA encoding TM1 was used and the membranes were washed at high stringency (0.1 × SSC at 65°C). To detect the low M_r TM isoforms TM4 (3.0 kb mRNA) and TM5 (2.3 kb mRNA), probes M1401 and M29, respectively, were used at the high stringency conditions [13,14]. We also used a probe, M1558, which recognizes TM1 and other muscle-type TM mRNAs. The northern blots were routinely stripped and reprobed with β-actin or GAPDH probes for load controls. The signals were quantified by a Molecular Dynamics phoshor imager (Typhoon 8600), and the ratios obtained with a specific probe and β-actin (or GAPDH) were calculated. These values represent a quantitative measure of the changes in the gene expression.

Cell lysates were prepared using Tris buffer containing 1% NP-40, 0.5% deoxycholate and a cocktail of protease inhibitors [12]. Two different antibodies were used to assess TM1 expression: a polyclonal antibody that recognizes multiple TM isoforms [7,12]; and, a TM1-specific antibody. A 45 amino acid region of TM1 (171–215 aa; [15]) was used to generate an anti TM1-specific antibody in rabbits. This antibody specifically recognizes TM1, but shows little or no reactivity against other TM isoforms. Fifty micrograms of cellular proteins were routinely analyzed in western blotting experiments. The blots were stripped and reprobed with anti α-tubulin antibody for load controls. After the chemiluminescence reaction, the exposed X-ray films were scanned and the signals were quantitated on the Typhoon imager. The ratios of the signal obtained with TM antibody and α-tubulin were used as a measure of changes in gene expression.

Immunofluorescence microscopy was performed essentially as described previously, using a TM antibody that recognizes multiple TM proteins [12]. Anchorage-independent growth assays were performed in soft agar according to the previously published procedures, except with the addition of AZA and TSA [8,10]. The drugs were added to the top agar at the concentrations indicated above, and the cultures were fed with or without (control) the appropriate drugs every 48 h. The samples were viewed with Olympus BX 60 microscope at 4× magnification (objective), and the individual colonies were enumerated.

The potential methylation islands on the TM1 gene were identified by GrailEXP v3.2 program (http://compbio.ornl.gov/grailexp).

3. Results and discussion

3.1. Isolation of TM1 gene

In breast carcinoma cells, TM1 expression is completely and consistently abolished. To investigate the mechanism of inhibition of TM1 expression, human TM1 gene was isolated by polymerase chain reaction (PCR) screening of a BAC library. A single BAC clone was isolated and the presence of TM1

gene was confirmed by exon specific PCR, restriction digestion mapping and partial sequencing. The BAC clone contained the entire TM1 gene consisting of 11 exons. TM1 is generated by alternate splicing from exons 1–6, 8, 9 and 11 [16]. The sequence information obtained was in agreement with the data deposited in GenBank (accession numbers: AF209746 and AL133410), and therefore, is not shown.

Computer analysis of the 5′ untranslated region (5′UTR) indicated the presence of three potential locations for methylation of cytosines residues in the CpG islands: −1252 to −861; −712 to −433; and, −204 to +117, with 'A' of initiation codon numbered as +1. These islands span a 1369 bp region in the 5′UTR through the first exon. Since the epigenetic silencing of many key genes is effected by promoter hypermethylation in cancers, including those originating in breast, we have examined whether promoter hypermethylation accounts for the loss of TM1 expression in breast cancer cells [11,17].

3.2. Hypermethylation and histone deacetylation of TM1 gene in cancer cells

Transcriptional silencing of many genes occurs during development and disease conditions, including in cancer. Gene methylation and chromatin remodeling allow reversible activation and inactivation of target genes both permanently as well as transiently [17]. Inactivation of the key regulatory genes either by mutation and/or epigenetic regulation results in the same biological consequence in that both processes disrupt normal regulatory circuits. Epigenetic silencing of many classical tumor suppressor genes such as APC, BRCA1, E-cadherin and Rb occurs in sporadic cancers, suggesting that hypermethylation is not a random event. Furthermore, many genes that are not fully documented as tumor suppressors, or important regulators, are inactivated by hypermethylation. It is likely that epigenetic regulation may be the primary mode of inactivation for some regulatory genes [17]. For example, HME1 (14-3-3σ) appears to be downregulated in breast tumor by promoter methylation [18].

Normal mammary epithelial cells express seven different TMs, among which TM1 is a prominent isoform that is also found in fibroblasts [7]. In breast cancer cells, TM1 expression is abolished, while the expression of other TMs varies. MCF-7 cells lack TM1, TM38 and TM2 isoforms, as shown by two-dimensional gel analyses [7]. We have used MCF10A cells as controls for normal mammary epithelial cells. It should be noted that MCF10A and MCF-7 are not isogenic cell lines, and therefore, only a qualitative comparison of TM expression profiles between the cell types is possible (Kalyankar et al., manuscript submitted). To investigate the mechanism of TM1 gene silencing, MCF-7 cells were treated with an inhibitor of DNA methyl transferase (DNMT), 5-aza-2′deoxycytidine (AZA) for 24 h, and RNA and protein were analyzed for TM1 expression, as described in Section 2. MCF-7 cells lack TM1 mRNA and protein (Fig. 1A,B). Addition of AZA to the cultures resulted in a modest induction of TM1-specific 1.1 kb mRNA and TM1 protein. The level of expression of TM1 attained with AZA alone was only barely detectable over the background. Prolonged culture with AZA for up to 9 days did not result in further enhancement of TM1 levels (data not shown).

Histones, in acetylated state, cause relaxation of tightly supercoiled chromatin leading to improved accessibility of DNA binding proteins and transcription factors to promoter region, culminating in gene transcription [19,20]. Histone deacetylases promote condensation of chromatin and gene silencing. Therefore, we examined whether TM1 expression is repressed by this mechanism. In fact, several genes, including estrogen receptor α and gelsolin are regulated by this mechanism [21,22]. Furthermore, inhibition of histone deacetylase (HDAC) activity suppresses the growth of breast cancer cells [23,24]. MCF-7 cells were incubated with trichostatin A (TSA), a well characterized inhibitor of histone deacetylation for 24 h and analyzed for TM1 expression. TSA treatment did result in a very small increase in TM1 expression (Fig. 1). Extended culture of MCF-7 cells with TSA alone up to 72 h did not induce TM1 to any higher levels (data not shown).

We next evaluated the combined effect of inhibition of DNMT and HDAC on TM1 gene expression. It has been postulated that gene methylation and histone deacetylation act as two layers in ensuring strong silencing of certain important genes in tumor development, and FMR1 gene which is mutated in fragile X syndrome [17,25,26]. Consistent with this notion, combined treatment of AZA and TSA resulted in robust reactivation of genes such as hMLH1,

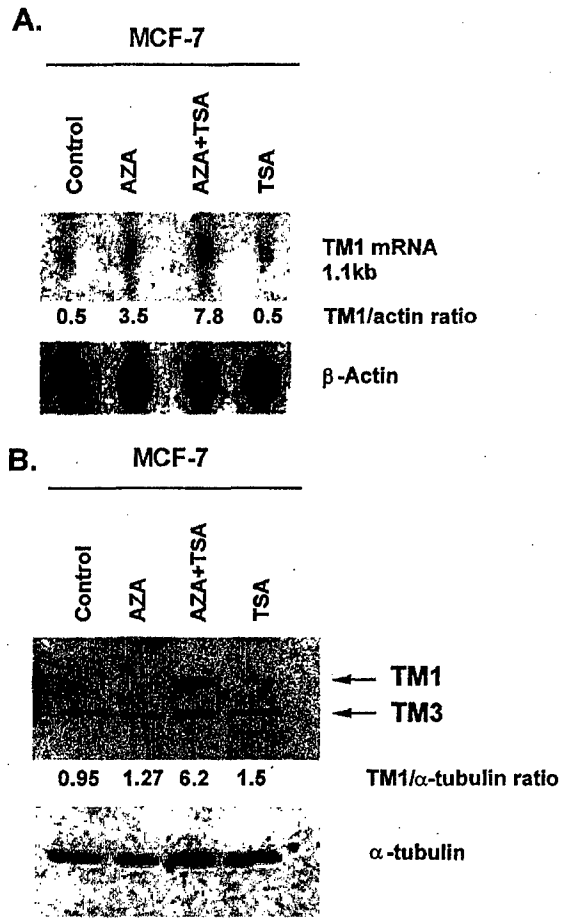

Fig. 1. Induction of TM1 by AZA and TSA. (A) Total RNA from the control MCF-7, AZA, TSA and AZA + TSA treated MCF-7 cells was probed for TM1 mRNA expression by a full length TM1 cDNA. For load controls, the same membrane was reprobed with β-actin probe. The ratio of TM1/β-actin signal is given. Control MCF-7 cells lack TM1 mRNA and therefore, the value indicates that of a background signal. (B) Immunoblotting of TM1 protein. Cellular extracts were analyzed by western blotting with a TM antibody. The positions of TM1 and TM3 are indicated. For load controls, the membrane was reprobed with anti-α-tubulin antibody. MCF-7 cells lack TM1 and the values represent background. TM1 is induced by a combination of AZA and TSA.

CDKN2A, TIMP3 and FMR1 [25,26]. Therefore, we tested whether AZA and TSA together would upregulate TM1 expression. Incubation of MCF-7 cells with AZA for 24 h followed by AZA and TSA for another 24 h (AZA total for 48 h) synergistically enhanced the expression of TM1 mRNA and the protein (Fig. 1A,B, respectively). This finding indicates that hypermethylation and chromosomal compaction by histone deacetylation are major mechanisms by which TM1 expression is silenced in breast cancer cells.

To investigate whether AZA and TSA mediated induction is specific to TM1, or the expression of all the TMs are generally upregulated by these drugs, mRNA levels of two low M_r TMs, were assessed. We utilized a cDNA probe, M1558 that is known to hybridize with several high M_r TMs to monitor the changes in the expression of TM1, TM2 and TM3 [13]. This probe showed the reactivation of TM1 gene in the cells treated with AZA and TSA together, but not other TMs. The results obtained with M1558 probe are identical to those described in Fig. 1A, and therefore, the data are not shown. Under the conditions of TM1 induction in MCF-7 cells, no significant change in the expression of TM4 and TM5 was detected by the drug treatment (data not shown). These data indicate that expression of TM genes is differently regulated, and that only TM1 expression is inactivated by gene methylation in breast cancer cells. Previous reports indicate that the changes in the expression of low M_r TMs is not often downregulated in cancer cells, and in some instances they are upregulated to compensate the loss of high M_r TMs [27].

3.3. Induced TM1 participates in microfilament assembly

TM1 is an important actin binding protein. Previous work from this laboratory showed that enhanced expression of TM1 results in microfilament reorganization in ras- and src-transformed fibroblasts, and in MCF-7 breast carcinoma cells [8–10]. To test whether the induction of TM1 expression by AZA and TSA would result in the incorporation of TM1 in microfilaments, immunofluorescence microscopy was performed (Fig. 2). The control MCF-7 cells lack the expression of TM1 and other TMs, and do not exhibit well-developed TM-containing microfilaments (panel a). However, MCF-7 cells do contain prominent actin filaments (panel b), possibly consisting of TM3, and other low M_r TMs (panel c). Similar results (data not shown) were obtained when the cells were treated with either AZA or TSA alone. This finding is consistent with the immunoblotting data which show a small increment in TM1 expression

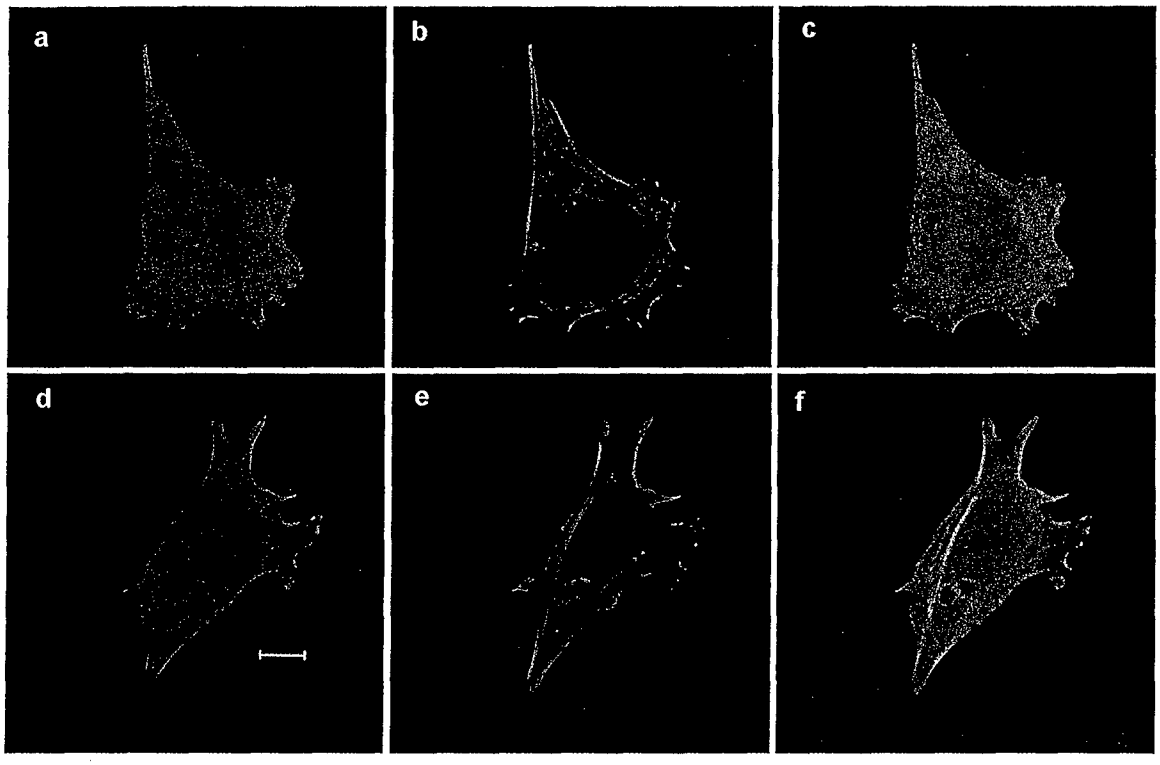

Fig. 2. Reorganization of microfilaments by induced TM1: control MCF-7 cells (panels a–c) and those treated by AZA and TSA (panels d–f) were stained with anti-TM antibody (a, d), or phalloidin (b, e). The merged images are shown in panels c and f. The drug treatment induces TM1 expression, and the induced TM1 participates in microfilament reorganization, as evident from d–f. The sizing bar indicates 10 μm.

with either AZA or TSA. When the cells were primed with AZA, and treated with TSA, the presence of TM1 containing microfilaments were clearly evident (panel d), and TM1 was colocalized with actin in those filaments (panels e and f).

3.4. Suppression of the transformed growth by AZA and TSA

Data from this laboratory show that TM1 is a suppressor of the malignant growth phenotype [8–10]. To determine whether AZA and TSA inhibit the anchorage-independent growth – a hallmark of the neoplastic growth, MCF-7 cells were cultured with AZA, TSA and together with AZA and TSA in soft agar. Control MCF-7 cells grew rapidly and formed colonies in soft agar (Fig. 3). The presence of AZA suppressed the colony formation appreciably (76%), while TSA alone was marginally (9.4%) effective. These data indicate that alterations in the expression of other key genes by AZA may contribute to the diminished growth in agar. In fact, key genes regulating growth such as retinoic acid receptor β2 and glutathione transferase P1 gene are activated by AZA treatment of MCF-7 cells [11]. Furthermore, methylation profiling of genes revealed that several genes are regulated by promoter methylation in breast cancer [28,29]. Reactivation of these genes or the tumor suppressor genes may be responsible for suppression of soft agar growth of AZA-treated MCF-7 cells. A more pronounced suppression (82%) in the anchorage-independent growth was accomplished in the presence of both AZA and TSA, confirming the synergistic action of these two drugs. A recent report [30] indicating that AZA and TSA synergistically upregulate estrogen receptor α gene and retinoic acid receptor β, and kill MDA MB231 cells, is in agreement with our finding that a combination of these two drugs is effective in abolishing the anchorage-independent growth.

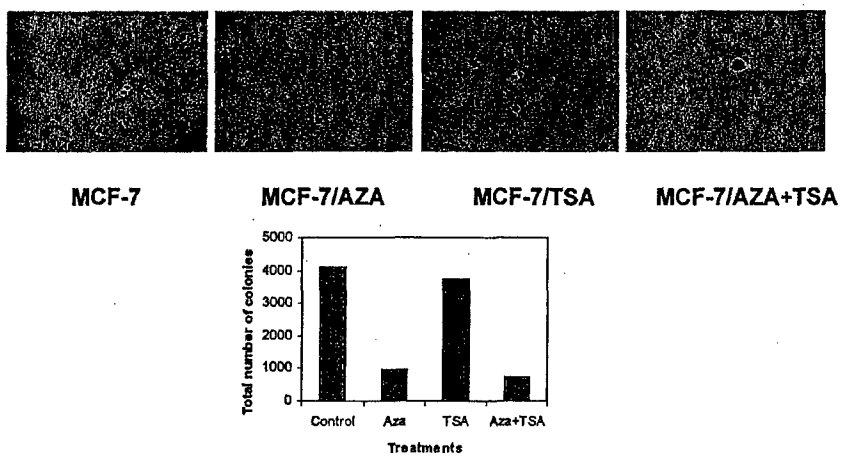

Fig. 3. Effect of AZA and TSA on the anchorage-independent growth. MCF-7 cells were cultured with either AZA, TSA and AZA + TSA, and counted, as described in Section 2. Photomicrographs of culture plates and the quantitative data are shown. Although AZA treatment decreases colony formation, the combined treatment of AZA and TSA is more effective in suppressing anchorage-independent growth.

3.5. Induction of TM1 expression in other cancer cells

To investigate whether gene hypermethylation and histone deacetylation account for the silencing of TM1 gene in other malignantly transformed cells, we assessed TM1 expression in two different cell lines. In MDA MB231 breast cancer cells, the 1.1 kb mRNA that codes for TM1 protein is lacking (Fig. 4A). As observed with MCF-7 cells, only the combined treatment of AZA and TSA reactivated the expression of TM1 mRNA. MDA MB231 cells also express an epithelial cell-specific TM isoform, TM38, which comigrates with TM1 (Fig. 4B, bottom panel) [7]. Therefore, an antipeptide antibody that recognizes TM1 with a high degree of specificity was used to assess TM1 expression in this cell line (Fig. 4B, top panel). Immunoblotting with this antipeptide antibody reveals the presence of TM1 in MCF10A cells, but not in MDA MB231 cells.

Treatment of cells with either AZA or TSA did not result in the restoration of TM1 mRNA or protein levels (Fig. 4A,B). Pretreatment of MDA MB231 cells with AZA, followed by addition of AZA and TSA, produced a significant induction of TM1, suggesting that inhibition of DNMT and HDAC are required for reactivation of TM1 expression. However, pretreatment of either of the breast cancer cell lines with TSA followed by addition of AZA did not upregulate the expression of TM1 (data not shown), supporting the notion that prior demethylation of TM1 gene may be a requirement for the chromatin decompaction by acetylated histones, and ultimately TM1 gene transcription [25]. Correspondingly, TM1 protein was detected in the cell lysates of the cultures treated with AZA and TSA (Fig. 4B, top panel). As observed with MCF-7 cells, culturing MDA MB231 cells with either of the drugs, or together did not result in significant changes in the expression of TM4 and TM5 (data not shown).

DT cells are ki-ras transformed NIH3T3 cells which are extensively used in this laboratory to study the role of cytoskeletal proteins in the cellular transformation [31]. The DT cells express TM1 at ≥50% of the levels found in NIH3T3 cells, and essentially undetectable levels of TM2 and TM3 [8,31]. In DT cells, TM1 RNA expression is downregulated to 50% levels found in NIH3T3 cells (Fig. 5A). In contrast to the breast cancer cells, AZA treatment produced a two-fold enhancement of TM1 mRNA, as evident from TM1/GAPDH ratios. TSA treatment, on the other hand, was less effective in upregulating TM1 mRNA. Once again, the combined treatment of AZA and TSA resulted in a further upregulation of TM1 mRNA (≥three-fold).

TM1 protein levels in DT cells are suppressed by about 65%, as measured by TM1/α-tubulin ratios, when compared to normal NIH3T3 cells (Fig. 5B), exceeding the 50% decrease in mRNA levels. AZA

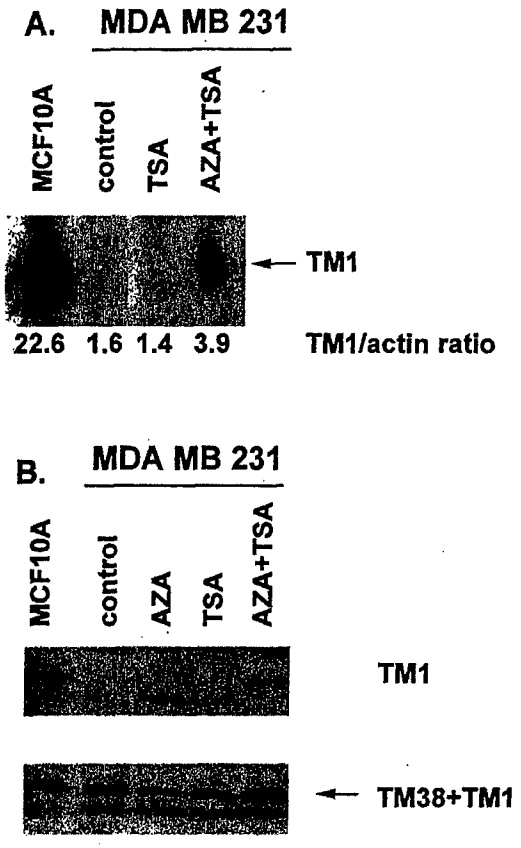

Fig. 4. Induction of TM1 in MDA MB231 cells by combined treatment of AZA and TSA. (A) Northern blotting. For comparison, RNA isolated from MCF10A cells is included. Control MDA MB 231 cells do not express TM1. (B) Immunoblotting of drug treated cellular lysates for TM1 expression. The MDA MB231 cells express an epithelial specific TM isoform, TM38, which comigrates with TM1. The polyspecific TM antiserum detects TM38 and TM1, and therefore the signal is present in both control and treated MDA MB231 cells (bottom panel). MCF10A cells express both TM1 and TM38. A TM1-specific antibody recognizes only TM1, but not TM38. Therefore, a signal is detected in MCF10A cells, but not in untreated MDA MB231 cells, or those treated with either AZA or TSA (top panel). TM1 expression, however, is induced by the combined treatment of AZA and TSA.

treatment of DT cells resulted in about 40% increase in TM1 expression compared to control DT cells. In addition, TSA treatment also enhanced TM1 expression to a much lesser, but to a detectable degree (12%). Combined treatment of DT cells with AZA and TSA produced further enhancement in TM1 protein levels (53% increase) compared to the control DT cells, underscoring the role of promoter methylation and chromatin conformation in regulating TM1 gene expression in tumor cells.

In addition to TM1, another high M_r TM isoform, TM2 was reactivated when treated with AZA and TSA (Fig. 5B). However, TM2 expression was substantially lower than in NIH3T3 cells. Reactivation of TM2 expression was not observed in MCF-7, or MDA MB231 cells, pointing to the differences in the regulation of TM proteins in different cancer cells. Thus, while TMβ gene is generally inactivated by gene methylation in cancer cells, it appears that down-regulation of TMα gene (which codes for TM2 and TM3) via promoter methylation may occur more specifically in ras transformation.

TM1 was suggested to belong to the class II tumor suppressors, which are transcriptionally silenced during the malignant transformation of cells [32]. Data presented in this communication indicate that promoter hypermethylation and chromatin remodeling by histones are primarily involved in silencing of TM1 gene in the malignant cells. However, there appear to be additional posttranscriptional mechanisms governing the expression of TM1, as evident from Fig. 5.

As discussed above, loss of TM1 expression is a common feature of many different malignant cells. Suppression of high M_r TMs, in particular that of TM1 and TM2 occur very rapidly, before the morphological transformation is evident, suggesting that the loss of TM1 may be an early event during tumorigenesis. Therefore, the loss of TM1 expression may serve as a potential biomarker. However, because of the high degree of sequence homology among the TMs, and a high degree of expression in the stromal and smooth muscle components of the tissue block has rendered the assessment of TM1 in human tumors difficult. Therefore, determination of the methylation status of TM1 gene may be useful in developing an approach to analyze and utilize TM1 expression as a prognostic indicator. Furthermore, since TM1 is a suppressor of the malignant growth, the induction of TM1 protein may contribute to the antineoplastic properties of AZA and TSA.

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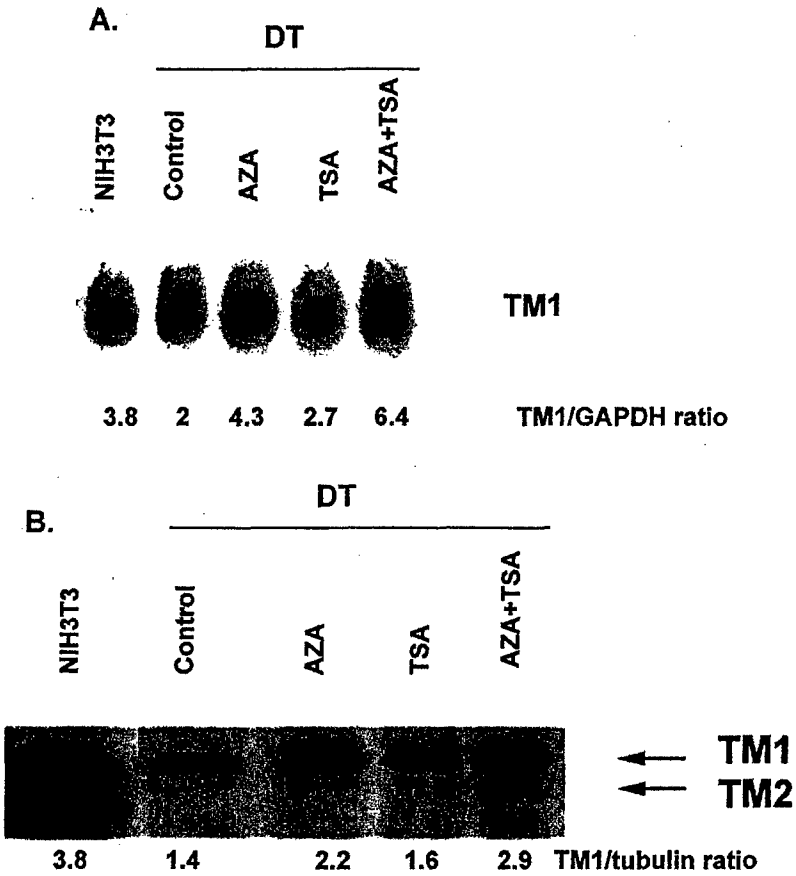

Fig. 5. Induction of TM1 expression in ras-transformed fibroblasts: (A) Northern blotting for TM1 expression. Total RNAs from NIH3T3, DT (ras-transformed NIH3T3 cells), along with DT cells treated with the drugs were isolated and probed with a full length TM1. The ratios of TM1/GAPDH signals are given. TM1 expression is induced significantly by AZA or AZA and TSA treatment. (B) Induction of TM proteins by AZA and TSA: cell lysates were immunoblotted using a polyspecific TM antibody. Treatment of DT cells with AZA significantly induced TM1 expression. However, the combination of AZA and TSA synergistically enhanced TM1 expression, and also reactivated TM2.

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References

[1] G. Pawlak, D.M. Helfman, Cytoskeletal changes in cell transformation and tumorigenesis, Curr. Opin. Genet. Dev. 11 (2001) 41–47.

[2] J.J. Lin, K.S. Warren, D.D. Wamboldt, T. Wang, J.L. Lin, Tropomyosin isoforms in nonmuscle cells, Int. Rev. Cytol. 170 (1997) 1–38.

[3] H.L. Cooper, B. Bhattacharya, R.H. Bassin, D.S. Salomon, Suppression of synthesis and utilization of tropomyosin in mouse and rat fibroblasts by transforming growth factor alpha: a pathway in oncogene action, Cancer Res. 47 (1987) 4493–4500.

[4] R.H. Warren, TGF-alpha-induced breakdown of stress fibers and degradation of tropomyosin in NRK cells is blocked by a proteasome inhibitor, Exp. Cell Res. 236 (1997) 294–303.

[5] S. Ljungdahl, S. Linder, B. Franzen, B. Binetruy, K. Sollerbrant, G. Auer, M.C. Shoshan, Down-regulation of tropomyosin-2 expression in c-Jun-transformed rat fibroblasts involves induction of a MEK1-dependent autocrine loop, Cell Growth Differ. 9 (1998) 565–573.

[6] R.A. Janssen, K.G. Veenstra, P. Jonasch, E. Jonasch, J.W. Mier, Ras- and Raf-induced down-modulation of non-muscle tropomyosin are MEK-independent, J. Biol. Chem. 273 (1998) 32182–32186.

[7] B. Bhattacharya, G.L. Prasad, E.M. Valverius, D.S. Salomon, H.L. Cooper, Tropomyosins of human mammary epithelial cells: consistent defects of expression in mammary carcinoma cell lines, Cancer Res. 50 (1990) 2105–2112.

[8] G.L. Prasad, R.A. Fuldner, H.L. Cooper, Expression of trans-

duced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene, Proc. Natl Acad. Sci. USA 90 (1993) 7039–7043.
[9] R.H. Braverman, H.L. Cooper, H.S. Lee, G.L. Prasad, Anti-oncogenic effects of tropomyosin: isoform specificity and importance of protein coding sequences, Oncogene 13 (1996) 537–545.
[10] G.L. Prasad, L. Masuelli, M.H. Raj, N. Harindranath, Suppression of src-induced transformed phenotype by expression of tropomyosin-1, Oncogene 18 (1999) 2027–2031.
[11] X. Yang, L. Yan, N.E. Davidson, DNA methylation in breast cancer, Endocr. Relat. Cancer 8 (2001) 115–127.
[12] V. Shah, S. Bharadwaj, K. Kaibuchi, G.L. Prasad, Cytoskeletal organization in tropomyosin-mediated reversion of ras-transformation: evidence for Rho kinase pathway, Oncogene 20 (2001) 2112–2121.
[13] A.R. MacLeod, C. Houlker, F.C. Reinach, L.B. Smillie, K. Talbot, G. Modi, F.S. Walsh, A muscle-type tropomyosin in human fibroblasts: evidence for expression by an alternative RNA splicing mechanism, Proc. Natl Acad. Sci. USA 82 (1985) 7835–7839.
[14] R.E. Novy, J.L. Lin, C.S. Lin, J.J. Lin, Human fibroblast tropomyosin isoforms: characterization of cDNA clones and analysis of tropomyosin isoform expression in human tissues and in normal and transformed cells, Cell Motil. Cytoskeleton 25 (1993) 267–281.
[15] G.L. Prasad, P.S. Meissner, D. Sheer, H.L. Cooper, A cDNA encoding a muscle-type tropomyosin cloned from a human epithelial cell line: identity with human fibroblast tropomyosin, TM1, Biochem. Biophys. Res. Commun. 177 (1991) 1068–1075.
[16] M.F. Pittenger, A. Kistler, D.M. Helfman, Alternatively spliced exons of the beta tropomyosin gene exhibit different affinities for F-actin and effects with nonmuscle caldesmon, J. Cell. Sci. 108 (1995) 3253–3265.
[17] S.B. Baylin, M. Esteller, M.R. Rountree, K.E. Bachman, K. Schuebel, J.G. Herman, Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer, Hum. Mol. Genet. 10 (2001) 687–692.
[18] C.B. Umbricht, E. Evron, E. Gabrielson, A. Ferguson, J. Marks, S. Sukumar, Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer, Oncogene 20 (2001) 3348–3353.
[19] S.Y. Archer, R.A. Hodin, Histone acetylation and cancer, Curr. Opin. Genet. Dev. 9 (1999) 171–174.
[20] B.M. Turner, Histone acetylation and an epigenetic code, Bioessays 22 (2000) 836–845.
[21] X. Yang, A.T. Ferguson, S.J. Nass, D.L. Phillips, K.A. Butash, S.M. Wang, J.G. Herman, N.E. Davidson, Transcriptional activation of estrogen receptor alpha in human breast cancer cells by histone deacetylase inhibition, Cancer Res. 60 (2000) 6890–6894.
[22] L.M. Mielnicki, A.M. Ying, K.L. Head, H.L. Asch, B.B. Asch, Epigenetic regulation of gelsolin expression in human breast cancer cells, Exp. Cell Res. 249 (1999) 161–176.
[23] D.M. Vigushin, S. Ali, P.E. Pace, N. Mirsaidi, K. Ito, I. Adcock, R.C. Coombes, Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo, Clin. Cancer Res. 7 (2001) 971–976.
[24] K. Schmidt, R. Gust, M. Jung, Inhibitors of histone deacetylase suppress the growth of MCF-7 breast cancer cells, Archiv der Pharmazie 332 (1999) 353–357.
[25] E.E. Cameron, K.E. Bachman, S. Myohanen, J.G. Herman, S.B. Baylin, Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer, Nat. Genet. 21 (1999) 103–107.
[26] P. Chiurazzi, M.G. Pomponi, R. Pietrobono, C.E. Bakker, G. Neri, B.A. Oostra, Synergistic effect of histone hyperacetylation and DNA demethylation in the reactivation of the FMR1 gene, Hum. Mol. Genet. 8 (1999) 2317–2323.
[27] J. Leavitt, G. Latter, L. Lutomski, D. Goldstein, S. Burbeck, Tropomyosin isoform switching in tumorigenic human fibroblasts, Mol. Cell. Biol. 6 (1986) 2721–2726.
[28] T.H. Huang, M.R. Perry, D.E. Laux, Methylation profiling of CpG islands in human breast cancer cells, Hum. Mol. Genet. 8 (1999) 459–470.
[29] P.S. Yan, M.R. Perry, D.E. Laux, A.L. Asare, C.W. Caldwell, T.H. Huang, CpG island arrays: an application toward deciphering epigenetic signatures of breast cancer, Clin. Cancer Res. 6 (2000) 1432–1438.
[30] V. Bovenzi, R.L. Momparler, Antineoplastic action of 5-aza-2′-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor beta and estrogen receptor alpha genes in breast carcinoma cells, Cancer Chemother. Pharmacol. 48 (2001) 71–76.
[31] H.L. Cooper, N. Feuerstein, M. Noda, R.H. Bassin, Suppression of tropomyosin synthesis, a common biochemical feature of oncogenesis by structurally diverse retroviral oncogenes, Mol. Cell. Biol. 5 (1985) 972–983.
[32] C. Sers, U. Emmenegger, K. Husmann, K. Bucher, A.C. Andres, R. Schafer, Growth-inhibitory activity and downregulation of the class II tumor-suppressor gene H-rev107 in tumor cell lines and experimental tumors, J. Cell Biol. 136 (1997) 935–944.

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APPENDIX 3

Suppression of the Transformed Phenotype of Breast Cancer by Tropomyosin-1

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Changes in the expression of microfilament-associated proteins, such as tropomyosins (TMs), are commonly found in malignantly transformed cells. Previous work from this laboratory has shown that tropomyosin-1 (TM1) expression is consistently abolished in human breast carcinoma cell lines, suggesting that the loss of TM1 could be a common biochemical event in the transformation of mammary epithelium. To investigate whether changes in TM1 expression are causally linked to mammary carcinogenesis, we have tested the hypothesis that TM1 is a tumor suppressor of breast cancer. MCF-7 cells, which lack TM1, were utilized as a model of human breast cancer and transduced to reexpress TM1 protein. Restoration of TM1 expression in MCF-7 cells (MCF-7/T cells) resulted in a slower growth rate, but cells remained sensitive to growth control by estrogen. TM1 expression in MCF-7 cells resulted in the emergence of TM-containing microfilaments. More significantly, MCF-7/T cells failed to grow under anchorage-independent conditions. TM1 reexpression alters the interaction of the E-cadherin–catenin complex with the cytoskeleton, indicating that TM1-induced cytoskeleton could play a significant role in suppression of the malignant phenotype. Taken together with our previous work on transformed murine fibroblasts, the results presented in this communication indicate that in nonmuscle cells TM1 functions as a suppressor of transformation.

Key Words: **tropomyosin; breast cancer; cytoskeleton; E-cadherin; β-catenin; tumor suppression.**

INTRODUCTION

During the neoplastic transformation, cells accumulate several different mutations and undergo extensive changes in gene expression. While the etiology of the vast majority of tumors is unknown, it is generally accepted that multiple genetic events contribute to the neoplastic transformation of cells [1]. Some of the changes include loss of tumor suppressor genes, activation of cellular proto-oncogenes, and inactivation/deregulation of the function or expression of key intracellular proteins. The mortality due to cancer is primarily a consequence of the ability of neoplastic cells to invade and metastasize in tissues where the cells do not normally grow. Tumor metastasis involves multiple steps including the loss of normal growth controls, the derangement of cytoskeletal organization, and the capacity to become motile and invasive [2–4]. It has been postulated that deregulation of growth factor (serum)-controlled and integrin-regulated adhesion pathways together contribute to the complete transformation involving accelerated proliferation and anchorage-independent growth [5]. In addition, tumor cells also manifest altered cell–cell adhesion and abnormal microfilaments, which facilitate invasion. Microfilaments are linked to both integrin and cadherin–catenin complexes which regulate cell–matrix and cell–cell adhesion, respectively.

While tropomyosins (TMs) have been known to function in regulation of muscle contraction, the functional significance of the multiple TM isoforms present in nonmuscle cells has remained largely unclear. Several reports indicate that nonmuscle TMs serve important functions in microfilament stabilization [6, 7], regulating microfilament branching [8], actin polymerization [9], modulation of myosin functions [10], and intracellular transport [11]. These reports strongly suggest isoform-specific functions for nonmuscle TMs. Our work on the role of cytoskeletal proteins in cell transformation has demonstrated that derangements in TM expression are a common biochemical change in many breast carcinoma cells [12]. This observation extended the earlier findings that loss of TM expression is commonly found in many experimentally transformed fibroblasts [13–18]. Furthermore, it was demonstrated, using oncogene-transformed murine fibroblasts that restoration of tropomyosin isoform 1 (TM1) expression

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is adequate to revert the malignant phenotype induced by functionally diverse oncogenes, such as *ras* and *src* [19–21].

TMs are a family of closely related actin-binding proteins [17, 22]. Multiple isoforms of TMs are expressed from four genes via alternate splicing in a highly tissue-specific manner. The 284 amino-acid high M_r TMs generally display higher affinity to actin than the low M_r isoforms (248 aa) [22]. Other important cytoskeletal proteins, such as caldesmon, fascin, and tropomodulin, also modulate TM interactions with actin [23, 24].

Although it is known that suppression of high M_r TMs is a prominent feature of many experimentally transformed murine cell lines, the relevance of TMs in human cancers is largely unknown. To that end, we have investigated the role of TMs in mammary carcinogenesis. In normal human mammary epithelial cells, seven different isoforms of TMs are expressed [12]. Among these, TM1, TM2, TM3, and an epithelial cell type-specific species, TM38, may be categorized as high M_r TMs. Isoforms TM32a, TM32b, and another epithelial-specific protein, TM32, are known to be low M_r TMs. In spontaneously transformed human breast carcinoma cell lines, loss of expression of multiple isoforms of TMs has been reported. More significantly, expression of TM1 is completely abolished in all the transformed cell lines, suggesting that suppression of TM1 could be a pivotal event leading to the acquisition of the neoplastic phenotype by mammary epithelial cells.

The experimentally transformed fibroblasts employed in the above studies to define a causal relationship of TMs to cell transformation are generated by a single well-defined transforming oncogene. Most human cancers, on the other hand, originate in epithelial cells as a result of multiple genetic defects. Furthermore, mechanisms that lead to transformation of epithelial cells could be different and more complex. For example, while both raf and ras transform fibroblasts, epithelial cells can only be transformed by ras [25]. Another complexity with epithelial cells is that at least two more TM isoforms are expressed in epithelial cells compared to fibroblasts, which could potentially compensate for loss of the other TMs. Therefore, it remains to be established whether TM1 can function as a suppressor of the malignant phenotype of spontaneously transformed, human-derived carcinoma cells. To further investigate the role of TM1 in cellular transformation, we determined whether restoration of TM1 expression in MCF-7 human breast cancer cells has an effect on the growth and transformation status of these cells.

MATERIALS AND METHODS

Normal mammary epithelial MCF10A cells were obtained from Dr. Jose Russo, Fox Chase Cancer Center, Philadelphia [26], and MCF-7 and MDA MB 231 cells were purchased from ATCC. The cDNA encoding TM1 protein has been previously described [27]. Anti-TM polyclonal antiserum which recognizes multiple TMs, including TM1, was described previously [12]. DT/TM1 and DT/TM1-TM2 cells were fibroblast cell lines and previously described [28, 29]. MCF10A cells are used in this study as a reference for TM expression. Since MCF10A and MCF-7 (or those derived from MCF-7 cells) are not isogenic, no direct comparisons on the growth properties of these cells are made.

For retroviral gene transfer of TM1 into MCF-7 and MDA MB 231 cells, a pBNC recombinant virus was used as described [19, 30], except that an amphotrophic packaging cell line PA317 was used to generate the infectious virus. TMe1 cDNA was subcloned into a pBNC retroviral vector in which a CMV intron/enhancer drives the expression of the gene of interest and the selection is accomplished with G418. Transduced MCF-7 cells were cloned by a limiting dilution method and the cell lines (MCF-7/T) were tested for TM1 expression. For control purposes, MCF-7 cells were transduced with the empty vector and the resultant cell lines were designated MCF-7/V cells. Cell lines derived from transduction of MDA MB 231 are referred as MDA MB 231/T cells.

Protein analysis. Two-dimensional gel electrophoresis using cell lysates prepared from metabolically labeled cells was performed essentially as described previously [12, 30]. Western blotting was performed with TM antiserum or commercially available antibodies. Expression of α-tubulin was routinely measured with a specific antibody (Sigma Chemical Co.) as a load control. Antibodies against E-cadherin and catenins were purchased from Transduction Laboratories. For routine analysis, cells were extracted with a 50 mM Tris buffer, pH 7.4, containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and a cocktail of protease inhibitors (1 mM PMSF and 1 μg/ml each of leupeptin, pepstatin, and aprotinin). After a brief centrifugation to remove nuclei and cell debris, the supernatant was used for immunoblotting or immunoprecipitations. These lysates were termed cytoplasmic preparations. Alternatively, cells were also extracted with RIPA buffer (RIPA lysates) [31]. To extract both nuclear and cytoplasmic proteins, cells were solubilized with 1% SDS and used for immunoblotting (whole cell lysates).

A 20-amino-acid sequence (187–206; SRARQLEEELRTMDQALKSL) that is distinctive for TM1 was used to generate a TM1-specific antibody in rabbits [27]. The antiserum was purified on a peptide immunoaffinity column and used for immunoblotting. It detects only TM1 on immunoblots. The polyclonal antiserum which recognizes all the TM isoforms has been previously described and was used for immunoblotting, immunocytochemistry and immunoprecipitations [28, 29].

Northern analysis. Total cellular RNA was analyzed as previously described [20]. The membranes were probed with a full-length TM1 cDNA [27]. The blots were washed at 65°C in a buffer containing 0.1× SSC and 1% SDS. Under these conditions, only TM1 is detected without any cross-hybridization to other TMs. Blots were then stripped and reprobed with β-actin for load controls.

Monolayer growth. Growth of the cells was measured in monolayer. Briefly, 2×10^5 cells were plated in normal (10%) serum containing media. At regular intervals, cells were harvested and counted using a hemocytometer. Cell culture conditions were previously described for MCF-7 and MDA MB 231 cells [26], and the medium for the transduced cells contained 200 μg/ml of G418. Experiments involving estrogen deprivation and supplementation were performed in a phenol red-free basal medium. Two replicate plates were initiated for each cell type and growth medium. To test the effects of estrogen on the growth of the cells, 72 h after plating, normal medium was replaced with a medium containing charcoal-stripped FBS alone (minus estrogen) or with a supplementation of 100 nM 17-βestradiol (plus estrogen). Cell count measurements were subsequently taken on each plate every 24 h (a total five measure-

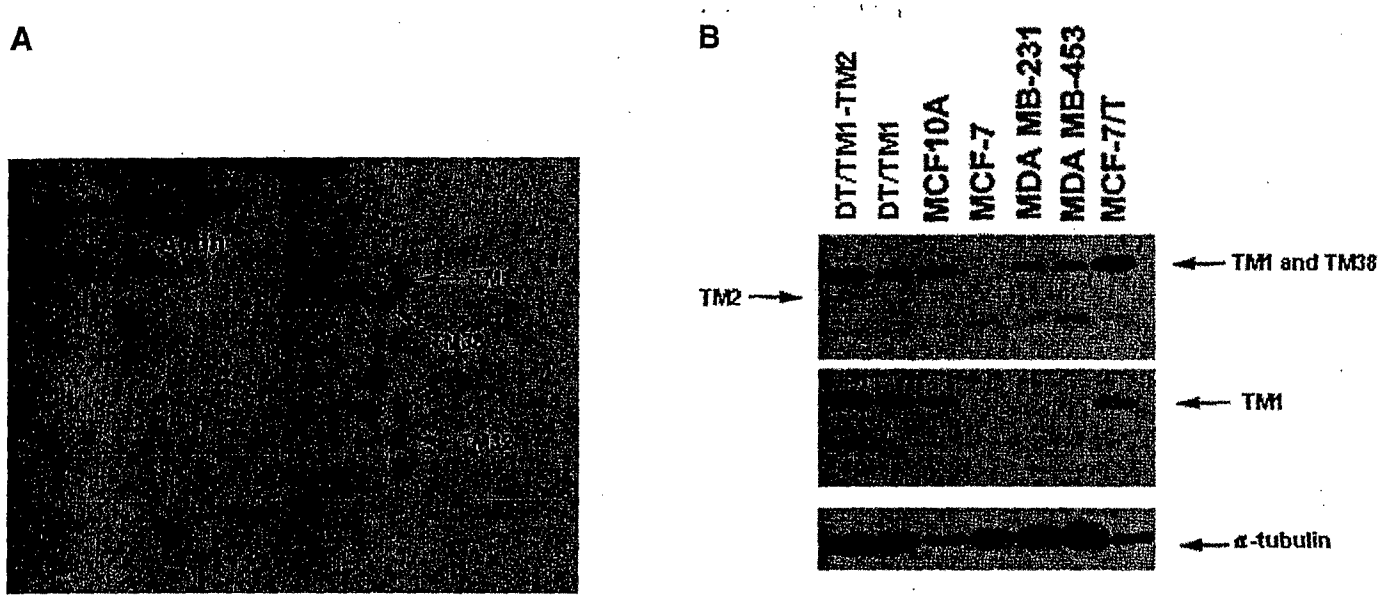

FIG. 1. TM expression in mammary epithelial cells. (A) Two-dimensional gel analysis of TM expression in MCF10A cells was performed as described under Materials and Methods. TM1, TM38, and TM32 are identified. (B) TM expression in normal and malignant breast cells. DT/TM1 and DT/TM1-TM2 cells were used as positive controls for TM expression. These are TM1-induced revertants of ras-transformed fibroblasts expressing either TM1 alone or both TM1 and TM2 [28]. TM2 migrates as a distinct band below TM1, which is evident in DT/TM1-TM2 cells (top). The cell lysates were probed with a polyclonal antiserum that reacts to multiple TMs (top), an antipeptide antibody that reacts specifically to TM1 (middle), or antitubulin antibody for load controls (bottom).

ments over 120 h). Mixed model analysis of variance (ANOVA) was performed, adjusting for estrogen exposure, to test for equality of growth after 120 h between the different cell types. Counts on the same plates were treated as a correlated measurements in the statistical analyses. Statistical analysis was performed using the SAS v8.1 software package (SAS, Cary, NC).

Anchorage-independent growth. Five or ten thousand cells were plated in soft agar in 35-mm petri dishes as described [19]. Cells were fed once in 48 h with 0.1 ml of medium. Six replicate plates were cultured for each cell type. Two weeks later, cells were stained with 0.05% nitroblue tetrazolium (Sigma). Colonies ≥ 50 μm were counted. Equality of the mean number of counted colonies after 2 weeks of incubation between the different cell types was tested using *t* tests.

Immunofluorescence. Cells were cultured in Nunc chamber slides, fixed with 3.7% paraformaldehyde [29], and permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS). For E-cadherin staining, samples were briefly extracted with 1% Triton X-100 and then fixed in paraformaldehyde, blocked, and reacted with primary antibody. The samples were blocked in 1% BSA (Jackson Immunolabs) in PBS and incubated with primary antibodies overnight at 4°C. The TM antiserum was used at 1:500, and other commercial antibodies were diluted according to the manufacturer's guidelines. The samples were also stained with Texas Red-conjugated phalloidin (Molecular Probes) for some experiments. The samples were mounted using the Prolong Antifade kit (Molecular Probes). Confocal microscopy was performed with a Zeiss confocal microscope with a 60X water objective. The images were optically sectioned and the composite images are projected.

For determination of the intensity of staining, the samples were viewed with a Zeiss Axioplan 2 microscope using either a 40X or 63X oil objective. The images were captured using a Dage MT1 camera (Model 300) and IFG 310 controller. The samples were photographed using different gate settings, which allows accumulation of different numbers of frames. The gate setting is inversely linked to the brightness of staining. For example, if a gate setting of 4 is used to photograph, four different individual frames will be taken and integrated in a final image. However, if a gate setting of 8 is required, it suggests that the image is about half as intensely stained as the first one. In these experiments, both the gain and black level, which affect the image quality, were kept at identical settings. The images were transferred to Adobe Photoshop and processed identically to make a composite image. Further quantification of the signal, such as area and intensity measurements using Photoshop, were not done. Multiple areas of the sample were photographed using gate settings of 4, 8, 16, and 32, depending on the intensity of the signal.

RESULTS

Restoration of TM1 expression in MCF-7 cells. Normal mammary and other epithelial cell types express seven different TMs, two more TMs than found in fibroblasts, that are readily detected in 2-D gels [12, 27]. TM expression in the nontransformed, immortalized human mammary epithelial MCF10A cell line is shown in Fig. 1A. One of these additional TMs, TM38, comigrates with TM1 in one-dimensional gels (Fig. 1B). The other TM isoform, TM32, is not resolved from the two low M_r TMs in routine SDS–PAGE, but requires 2-D analyses. Neither of these TM isoforms is well characterized. In malignant breast epithelial cells, TM1 expression was consistently absent, but the expression of other TMs including that of TM38 varies. For example, in MDA MB 231 and MDA MB 453 cells, TM38 expression is detectable by immunoblotting with polyclonal anti-TM antisera and by 2-D gels (Fig. 1B, top; [12]). However, in MCF-7 cells both TM1 and TM38 are lacking, and therefore no signal is detected.

The fibroblast cell lines DT/TM1 and DT/TM1-TM2, which express either TM1 alone or TM1 and TM2 together, respectively, are used as positive controls for TM1 expression. These cells lack the expression of TM38, and hence, the signal in the fibroblast-derived cell lines represents the expression of TM1 [12, 27, 28].

To facilitate the analysis of TM1 expression, we generated a specific antipeptide antiserum, as described under Materials and Methods. Data from immunoblotting with TM1-specific antibody are shown in Fig. 1B (middle). The two fibroblast cell lines DT/TM1 and DT/TM1-TM2 and the MCF10A cells contain readily detectable TM1. In agreement with two-dimensional gel analyses, expression of TM1 was lacking in MCF-7, MDA MB 231, and MDA MB 453 breast cancer cell lines [12]. In MDA MB 231 and MDA MB 453 cells which express TM38, no signal was detected by TM1-specific antibody, indicating the lack of TM1. MCF-7 cells that were transduced to reexpress TM1, designated MCF-7/T, however, were positive for TM1 expression (discussed below).

In MCF-10A human mammary epithelial cells, TM1 is expressed from a 1.1-kb mRNA. In MCF-7 human breast carcinoma cells, expression of both TM1 protein and its cognate mRNA is totally abolished, as is the case with several other breast carcinoma cells (Fig. 2A; also Fig. 1B) [12]. Transduction of MCF-7 cells with a recombinant retroviral vector results in the expression of a 2.0-kb mRNA from which TM1 is transcribed, as found in MCF-7/T cell lines; TM1 mRNA is 1.1 kb in size, while the additional sequences originate from the vector [19]. We have analyzed three independent MCF-7/T cell lines and two MCF-7/V cell lines along with the parental MCF-7 cells. In MCF-7/V cell lines (vector controls) and the parental MCF-7 cells, the transduced 2.0-kb mRNA is absent, as expected.

Immunoblotting of cytoplasmic lysates with a polyclonal anti-TM antibody revealed that TM1 is readily detectable in MCF10A cells. However, no corresponding signal was present in MCF-7 or MCF-7/V cells. In MCF-7/T cell lines, TM expression is restored (Fig. 1B, middle; data not shown for other cell lines). Since high M_r TMs do not resolve well in one-dimensional gels and the individual TMs significantly vary in their avidity to antibody, we examined total expression of TMs in these cell lines. To that end, cellular extracts prepared from metabolically labeled cells were analyzed by two-dimensional gels (Fig. 2B). In MCF-7 cells, expression of TM1, TM38, and TM2 is abolished, and therefore, none of these three proteins are detected [12]. Among the muscle-type high M_r TMs that are present in epithelial cells, only TM3 is present in MCF-7 and MCF-7/V cells. Transduction of MCF-7 cells with TM1 cDNA, as expected, results in a specific enhancement of TM1 protein in MCF-7/T cells. Furthermore, transduced TM1 is also found in the cytoskeletal fraction of MCF-7/T cells (data not shown).

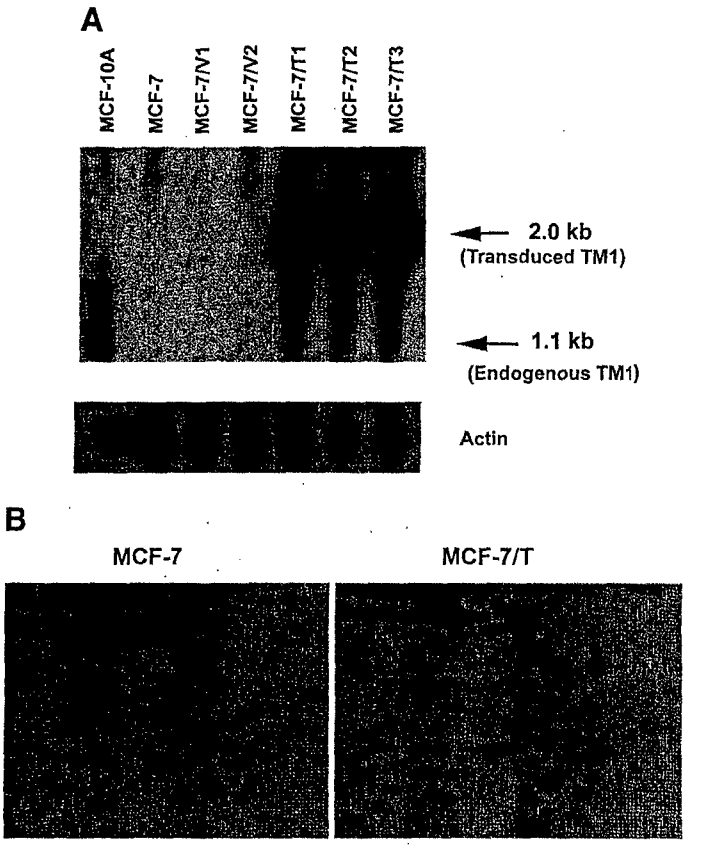

FIG. 2. Tropomyosin expression. (A) Northern blotting: Total cellular RNAs were probed with a full-length cDNA encoding TM1 at high stringency. Transduced TM1 is expressed as a 2.0-kb mRNA, while the endogenous TM1 is synthesized as a 1.1-kb RNA. The parental MCF-7 cells lack the mRNA encoding TM1. β-Actin was used for load controls (bottom). (B) Two-dimensional gel analyses of TM expression. Total cell lysates were prepared from pulse-labeled cells and analyzed by two-dimensional gels. TM1 is identified in the right panel.

Morphology of MCF-7/T cells. Restoration of TM1 expression in MCF-7 cells resulted in significant morphological changes. MCF-7 and the vector control cells shows that they grow as rather loosely adhering clusters. MCF-7/T cells in general grow in tighter clusters and form distinctive tubular structures (Fig. 3A) [32].

Immunocytochemical staining with anti-TM antibody of parental MCF-7 cells showed weak staining with the TM antiserum, although MCF-7 cells express at least one high M_r TM isoform and low M_r TMs. TM staining in MCF-7 cells is faint and diffuse throughout the cell, with no detectable association with actin filaments (Fig. 3B; A–C). In MCF-7/T cells, TM staining was intense, and TM containing microfilaments were evident. In addition, TM staining colocalized with that of actin, indicating that transduced TM1 reorganizes microfilaments (Fig. 3B; D–F). Although TM staining

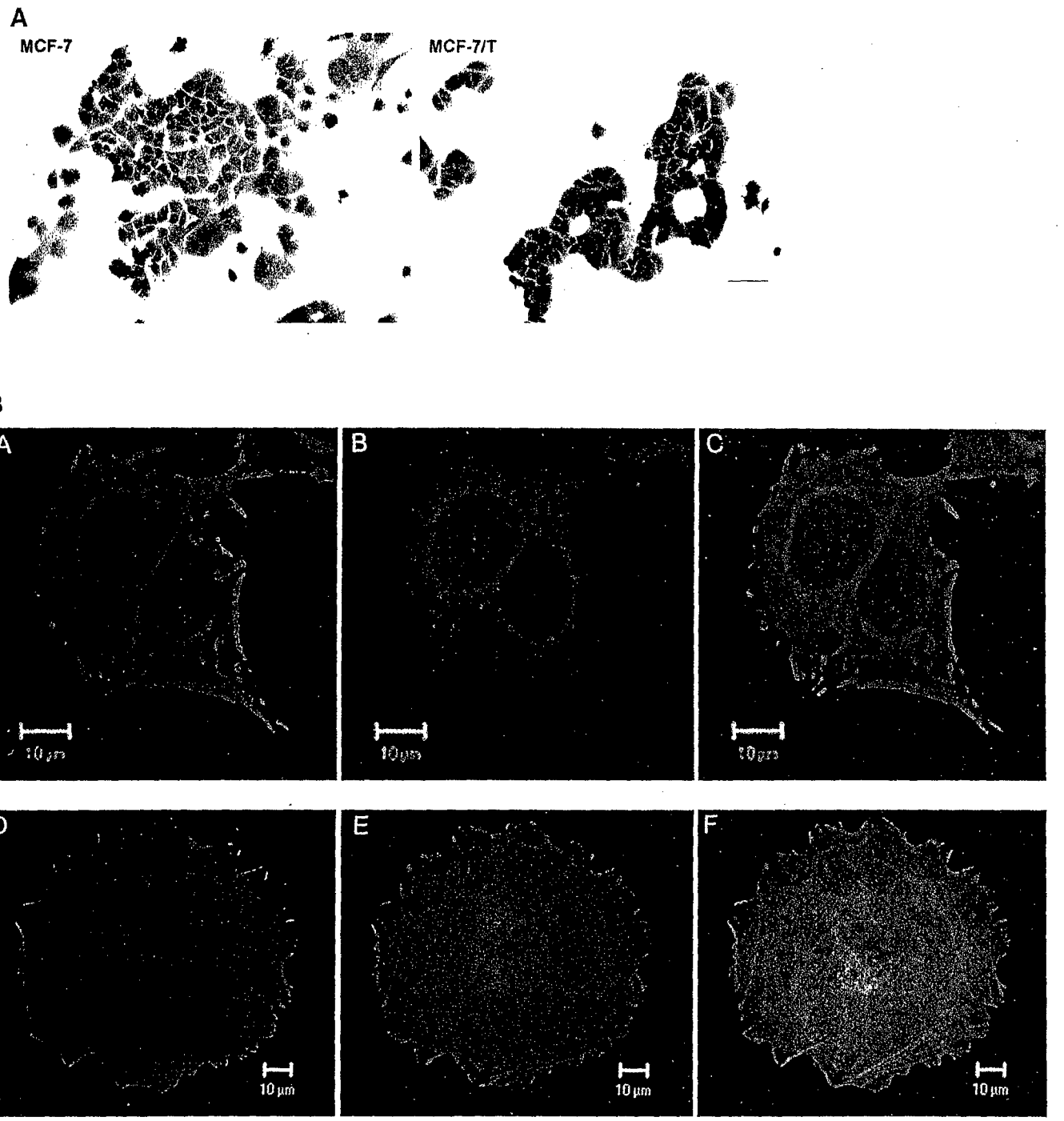

FIG. 3. (A) Morphology of TM1-expressing MCF-7 cells. Monolayers of MCF-7 and MCF-7/T cells were stained with H & E and photographed using an Olympus B20 microscope with a 2X objective. (B) TM1 associates with microfilaments. MCF-7 (A–C) and MCF-7/T (D–F) cells were immunostained with TM antiserum (A and D) followed by binding to FITC-conjugated antirabbit antibody (green) and Texas Red-conjugated phalloidin (B and E; red). Merged images (C and F) are presented. The samples were viewed using a confocal microscope.

was found through the cell body, it was brightest around the nucleus.

Growth properties. The effect of TM1 expression on the growth properties of MCF-7 cells was assessed in monolayer cultures. MCF-7, a vector control cell line (V1), and three cell lines expressing TM1 (T1, T2, and T3) were used to measure the growth. Under normal serum conditions, the unmodified MCF-7 and those transduced with empty vector grew rapidly at similar rates (Fig. 4A). However, all the three individual cell

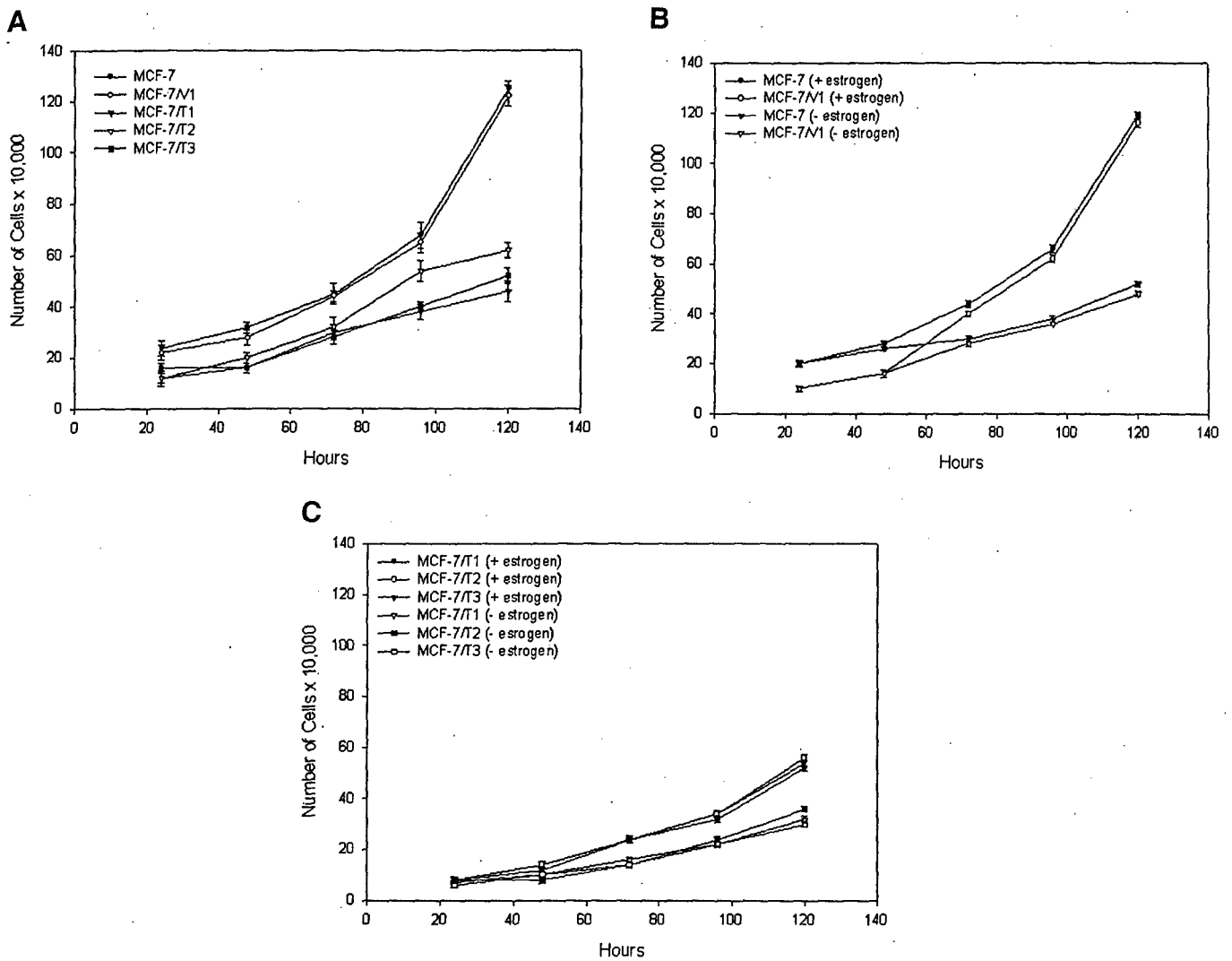

FIG. 4. TM1 expression decreases growth of MCF-7 cells. Cells were cultured under normal growth medium (A), in charcoal-stripped medium (estrogen deprivation), or in charcoal-stripped serum supplemented with 100 nM estradiol. The growth of MCF-7 and MCF-7/V (B), the three MCF-7/T cell lines (C), and under conditions of estrogen deprivation and supplementation is depicted. Error bars indicate standard deviation. The revertant cells grew significantly more slowly than the parental MCF-7 or vector control cells in normal serum, in the absence of or under estrogen supplementation.

lines expressing TM1 grew comparably, but demonstrated strikingly slower growth after 120 h than the parental MCF-7 or MCF-7/V cell lines ($P < 0.0001$ for all pairwise comparisons). Thus, restoration of TM1 expression decreases the proliferation of breast carcinoma cells.

Estrogen regulates the growth and differentiation status of MCF-7 cells. Since TM1 expression decreases the growth of these cells, we tested whether MCF-7/T cells remain sensitive to growth control by estrogen (Figs. 4B and 4C). When cells were cultured in the absence of estrogen using charcoal-stripped fetal bovine serum, growth of all the cell lines, including those expressing TM1, was inhibited by about 50%. Even under these conditions, MCF-7 and MCF-7/V1 cells (Fig. 4B) maintained higher growth than MCF-7/T cells, as shown in Fig. 4C ($P < 0.0001$ for all pairwise comparisons). Supplementation with 100 nM β-estradiol in charcoal-treated serum containing medium resulted in increased growth. In the presence of estrogen, MCF-7 and MCF-7/V1 cells demonstrated profoundly enhanced growth compared to MCF-7/T cells ($P < 0.0001$ for all pairwise comparisons). Addition of 4-hydroxytamoxifen inhibited the stimulatory effect of estrogen (data not shown). These data show that restoration of TM1 expression decreases the growth of MCF-7 cells, without altering the sensitivity to estrogen.

Anchorage-independent growth. The proliferation of normal cells is tightly regulated by growth signals of

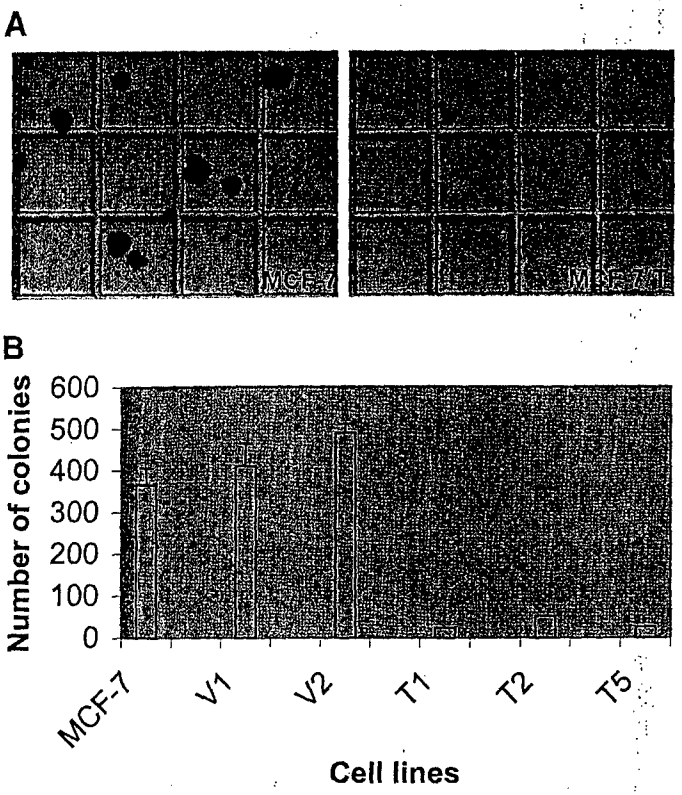

FIG. 5. TM1 suppresses anchorage-independent growth. Cells were plated in soft agar as described under Materials and Methods. At the end of the culture, they were stained with nitroblue tetrazolium, and photographed (A) and the number of colonies formed with each cell line is shown (B). Error bars indicate standard deviation.

integrin–extracellular matrix interactions, which is often deregulated in tumor cells. The ability of tumor cells to proliferate independent of adhesion closely correlates with tumorigenic potential, which is often assayed by anchorage-independent growth in soft agar. The data presented in Fig. 4 show that reexpression of TM1 decreases the growth of MCF-7 cells. Therefore, we examined whether TM1 inhibits anchorage-independent growth of MCF-7 cells. MCF-7 and V1 and V2 cells grew rapidly and formed a large number of colonies within 2 weeks (Figs. 5A and 5B). In contrast, the three clones of MCF-7 cells expressing TM1 did not grow in parallel cultures. The MCF-7/T cell lines showed a significant decrease in growth in the number of colonies ($P < 0.0001$ for all comparisons between MCF-7, V1, and V2 cells and MCF-7/T cells). These data demonstrate that reexpression of TM1 abolishes the anchorage-independent growth of breast carcinoma cells and support our earlier studies on the antitransformation effects of TM1.

Cell–cell adhesion molecules in TM1-transduced breast cancer cells. Expression of E-cadherin is downregulated in several human malignancies, including breast cancer. Decrease in E-cadherin expression weakens cell–cell interactions and contributes to the metastatic phenotype. Additionally, E-cadherin complexes with α, β, and γ catenins, and the entire complex is anchored to microfilaments. The cadherin–catenin complex is implicated in regulating tissue integrity, polarity, and differentiation. Stable association of the cadherin–catenin complex to microfilaments is considered a requirement for normal functioning of cadherin complexes [33, 34]. Furthermore, free, soluble β-catenin that is not associated with cadherins, is a key player in the wnt signaling pathways [35, 36]. Activation of wnt signaling pathways results in the transportation of β-catenin into the nucleus and interaction with TCF/LEF transcription factor, thereby upregulating gene expression. Since cells expressing TM1 form tighter clusters and display a tubular morphology indicating enhanced differentiation, we investigated whether TM1-induced reversion of breast carcinoma cells involves changes in the expression of E-cadherin or the catenins.

Cytoplasmic lysates were prepared from actively growing cells and were immunoblotted with antibodies against E-cadherin and α, β, and γ catenins. The data of Fig. 6A indicate that MCF-7 and the two vector control cell lines express similar levels of all the four proteins tested. The three MCF-7/T cell lines, however, showed significantly and consistently lower levels of E-cadherin and the catenins (Fig. 6A). Since TM1 expression is associated with the reemergence of microfilaments, we investigated a possibility that the cadherin complex may be more tightly associated with the cytoskeleton in the revertants, thus, forming stronger cell–cell junctions which may make them less soluble. To examine the total expression of these proteins, cells were extracted under more vigorous conditions, using RIPA buffer to lyse the cells, and tested for the presence of the components of the cadherin–catenin complex. Under these conditions, no detectable differences in the expression of E-cadherin or the catenins were found between the transformed (MCF-7 and V) and the revertant TM1-expressing MCF-7/T clones (Fig. 6B): similar results were obtained when cells were solubilized with 1% SDS and lysates were analyzed (data not shown). These results indicate that in MCF-7/T cell lines, E-cadherin and the catenins associate more tightly to the cytoskeleton, presumably contributing to the stability of cell–cell adherens junctions.

To investigate whether the localization of E-cadherin and the catenins is altered in the TM1-expressing cells, immunocytochemistry was performed. In MCF-7 cells, β-catenin (Fig. 7A; A) and E-cadherin (Fig. 7A; B) were detectable at the cell–cell junctions. In MCF-7 cells, E-cadherin's presence was evident in the perinuclear area as well as in the cytoplasm. In the revertant MCF-7/T cells, E-cadherin and β-catenin were also readily detectable at the cell adhesion areas with well-

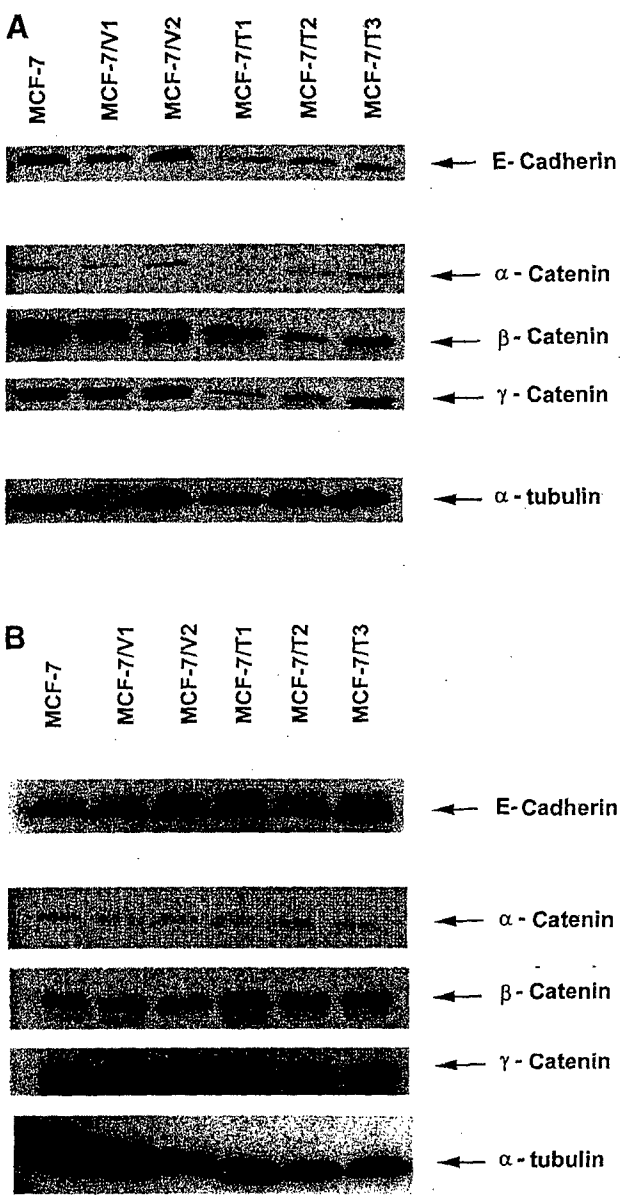

FIG. 6. The E-cadherin–catenin complex is more tightly associated in MCF-7/T revertant cells. Cytoplasmic lysates (A) and RIPA lysates (B) of the indicated cells were analyzed for expression of E-cadherin and the catenins by immunoblotting. Note that in the RIPA extracts, E-cadherin and the catenins are detected at comparable levels between the transformed and the revertant lines of MCF-7 cells.

defined staining (Fig. 7A; C and D, respectively). Further detailed analysis of the organization of these two molecules in the revertant cells revealed significant differences from the parental MCF-7 cells.

While both parental MCF-7 and MCF-7/T cells contained E-cadherin and β-catenin at cell–cell junctions, their association with the detergent-insoluble fraction was different between the cell types. The samples were stained with respective antibodies, visualized using a Zeiss fluorescence microscope, and photographed for different exposures as described under Materials and Methods. The samples were exposed to accumulate different number of frames, without any other adjustments to either black level or brightness settings. It was found that MCF-7 cells had significantly lower detergent-resistant E-cadherin and β-catenin at the cell–cell junctions (Fig. 7B, a and b) vis-à-vis MCF-7/T cells (Fig. 7B, c and d). When the samples were photographed for the same number of frames, the intensity of either E-cadherin and β-catenin at the cell–cell junctions was higher in MCF-7/T cells. MCF-7/T cells consistently retained higher amounts of E-cadherin and β-catenin at the cell–cell junctions than in MCF-7 cells. Multiple areas of both the cell types were examined at more than two gate settings. These data suggest that upon transduction of TM1, E-cadherin and β-catenin are more tightly linked to the cytoskeleton, which is in line with the immunoblotting results (Fig. 6). Under these conditions, α and γ catenins, however, were found to be at comparable levels in MCF-7 and MCF-7/T cells (data not shown).

Effects of restoration of TM1 on other breast cancer cells. TM1 expression is lacking in several other breast carcinoma cell lines [12]. To test whether TM1 functions as a suppressor in other breast carcinoma cell lines, or the anti-transformation effects of TM1 are limited to MCF-7 cells, we utilized the widely studied MDA MB 231 cells. MDA MB 231 cells are estrogen receptor-negative cells and highly invasive cells with fibroblastic features [37]. MDA MB 231 cells were transduced with TM1 and several cell lines (MDA MB 231/T cells) were isolated. Figure 8A shows immunoblotting with TM1-specific antibody and a polyclonal TM antibody of parental and transduced MDA MB 231 cell lysates. In agreement with the data of Fig. 1B, MDA MB 231 cells express only TM38, but not TM1, and accordingly a signal corresponding to TM38 was detected (Fig. 8A, middle). In two separate clones of MDA MB 231/T cells, transduced TM1 is detected with TM1-specific (Fig. 8A, top) and polyclonal antibody (Fig. 8A, middle). For comparison, we included MCF-7 and MCF-7/T1 cell lines, as TM1 is present in the latter cell line.

Next, we examined whether TM1 suppresses the anchorage-independent growth of MDA MB 231 cells. Two independent cell lines expressing TM1 and unmodified MDA MB231 cells were plated in soft agar and the colony formation was examined (Fig. 8B). The MDA MB 231 cells grew very rapidly and formed large colonies within 2 weeks, while the TM1-transduced cells did not show any growth even after 3 weeks in culture. These data indicate that restoration of TM1 expression is adequate to abolish a key attribute of transformed phenotype of MDA MB 231 cells. These

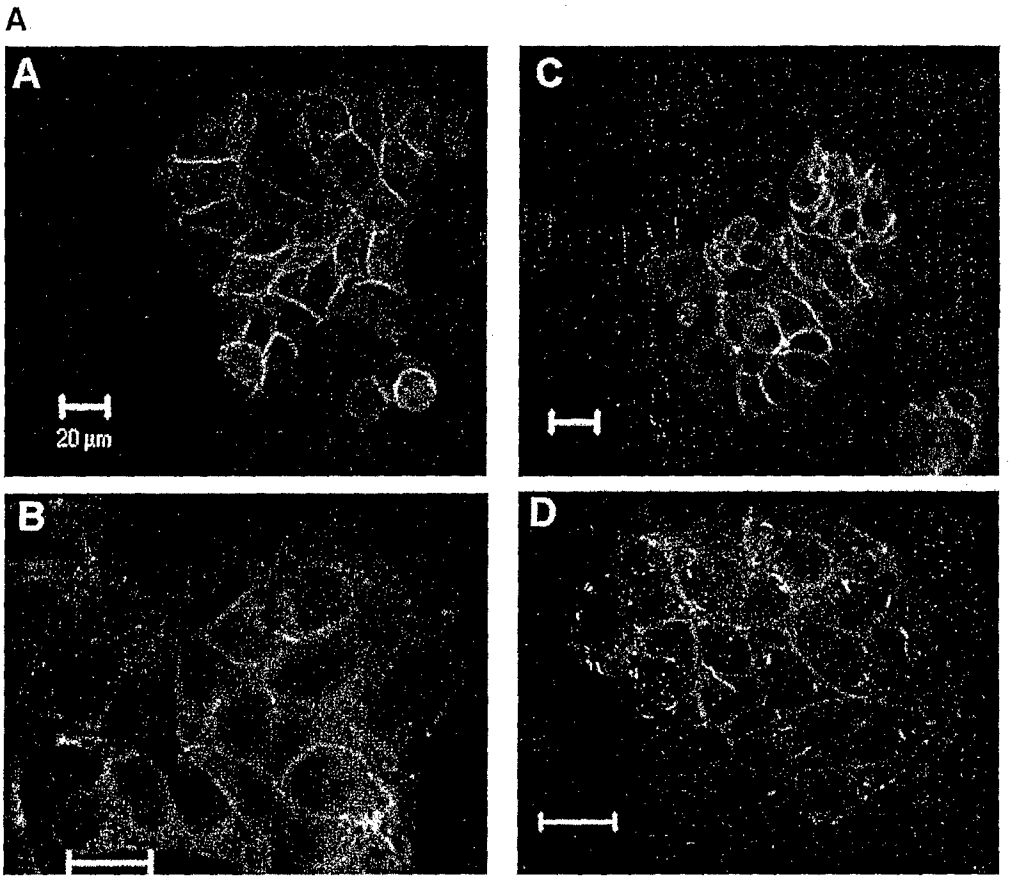

FIG. 7. (A) Organization of E-cadherin and β-catenin. MCF-7 (A and B) and MCF-7/T (C and D) cells were stained with either E-cadherin (A and C) or β-catenin (B and D) and examined by immunocytochemistry. In both cell types, the cell adhesion molecules are found at the cell–cell junctions. (B) E-cadherin and β-catenin are tightly associated at the cell–cell junctions of MCF-7/T cells. MCF-7 (a and b) and MCF-7/T (c and d) cells were stained with either E-cadherin (a and c) or β-catenin (b and d). The samples were exposed at different gate settings to accumulate different numbers of frames, which is dependent on the intensity of the signal. E-cadherin-stained samples were photographed at 16 frames, and β-catenin at 8 frames. Images at other higher or lower settings are not shown.

findings further support the hypothesis that TM1 is a suppressor of neoplastic transformation.

DISCUSSION

It is now widely recognized that normal functioning of the cytoskeleton is essential for maintaining normal growth and differentiation. For example, actin microfilaments are important determinants of cell morphology, cell motility, cell polarity, and cell division. In addition, reorganization of actin microfilaments occurs in response to activation of integrins and it is likely to play an important role in "inside out" signaling [5]. The attachment of cadherin–catenin complexes to microfilaments is known to strengthen cell–cell adhesion and contribute to tissue integrity. Furthermore, more recent data suggest that many signaling molecules are linked to microfilaments [38, 39]. Transformed cells generally lack well-defined microfilament bundles which, besides being a cause for loss of normal morphology, are potentially important contributing factors for metastatic behavior [3].

Several lines of evidence presented herein support the thesis that TM1 is a suppressor of the transformed phenotype of breast cancer cells: (i) restoration of TM1 expression results in the growth of MCF-7 cells as tighter colonies with a more glandular morphology, while the parental and wild-type MCF-7 cells grow as more scattered colonies; (ii) TM1 expression leads to significantly decreased growth in monolayer cultures; and (iii) TM1 expression completely abolishes the anchorage-independent growth of two spontaneously transformed breast cancer cells, viz. MCF7 and MDA MB 231 cells. Earlier work from this laboratory has demonstrated that TM1 reverts the transformed phenotype of *Ki-ras-* or *v-src*-transformed fibroblasts. Taken together, these findings support the notion that TM1 could be a general suppressor of malignant transformation.

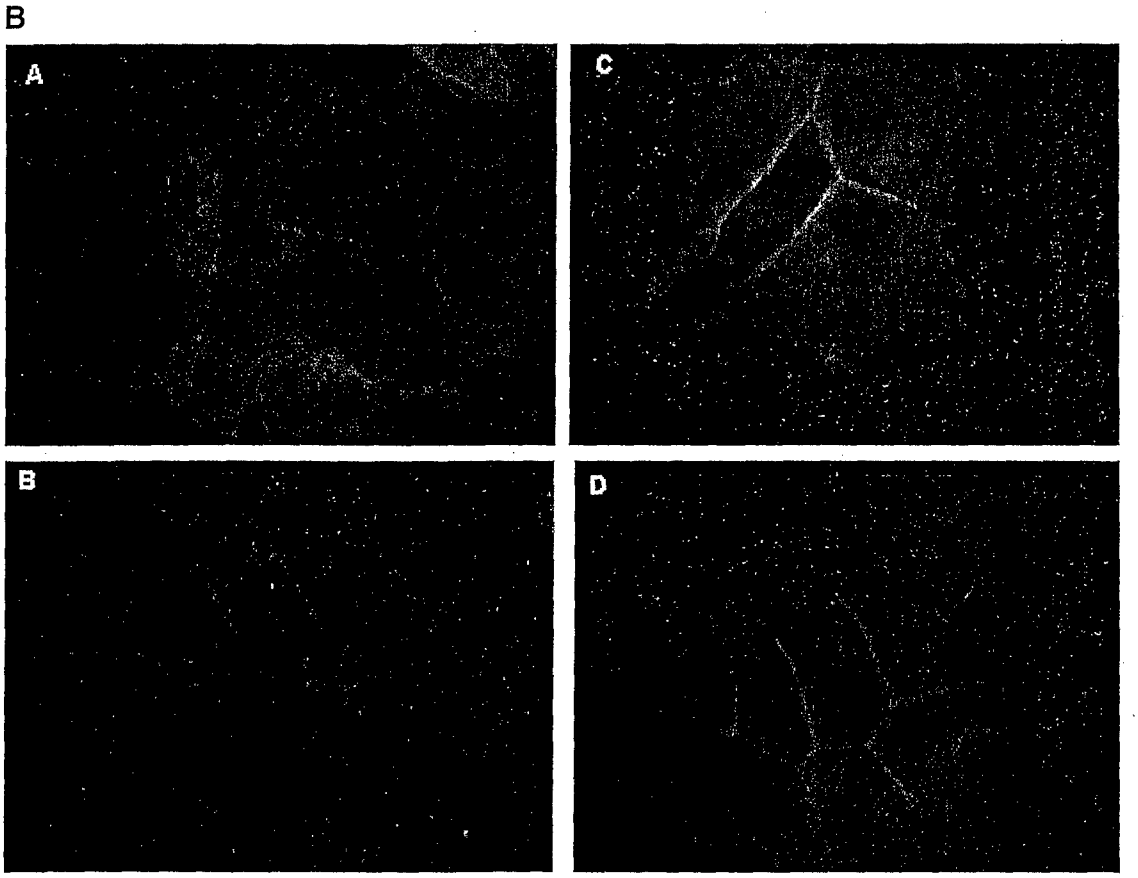

FIG. 7—*Continued*

Many key questions, however, remain regarding the role of TM1 in malignant transformation. First, although TM1 expression has been shown to be downregulated or lacking in many tumor cell lines, it is not clear whether the expression of TM1 is lost in primary tumor tissues. A main reason for this gap in understanding the role of TM1 in cellular transformation is the lack of suitable reagents to assess TM1 expression specifically in tissues where multiple TM isoforms are expressed. The downregulation of TM1 expression in breast and other cancer cells appears to be mediated by TM1 promoter methylation and histone deacetylation [40].

Second, the mechanism of TM1-mediated suppression of transformed growth is poorly understood. TM1 is a structural protein and does not possess the functional domains that may mediate specific protein–protein interactions, which could readily explain its role as a suppressor of transformation. TM1, like other TMs, is an actin-binding protein, and we find that TM1 alone restores microfilament architecture and suppresses the transformed growth phenotype: other TMs, such as TM2, cooperate with TM1 in reorganizing the cytoskeleton [28]. We investigated the possibility that the TM1-induced cytoskeleton could be important in the reversion of transformed growth.

Cadherin–catenin complexes mediate cell–cell adhesion and a number of studies have demonstrated that the integrity and functioning of these cell adhesion complexes are disrupted in many types of cancers, including those originating in breast [41–43]. E-cadherin, often referred to as a metastasis suppressor, is either generally expressed at low levels [2, 44] or mutated in some breast cancer specimens [45, 46]. The interactions of cadherin–catenin complexes with the cytoskeleton are important in maintaining normal adhesion via cadherins [33, 34].

In TM1-expressing revertants of MCF-7 cells, while there appear to be no qualitative differences, E-cadherin and the catenins are more tightly coupled to cytoskeleton, as evident from the differential extractability of these proteins. Enhanced detergent solubility of E-cadherin and β-catenin is more frequently observed in transformed cells, which may result in the assembly of defective adhesion junctions. In TM1-expressing revertant cells, E-cadherin and β-catenin are more tightly associated with the cytoskeleton, as evidenced by immunoblotting studies (Figs. 6 and 7) and immunofluorescence experiments. Consistent with these data, it was reported that ras transformation of breast epithelial cells does not change the expression of

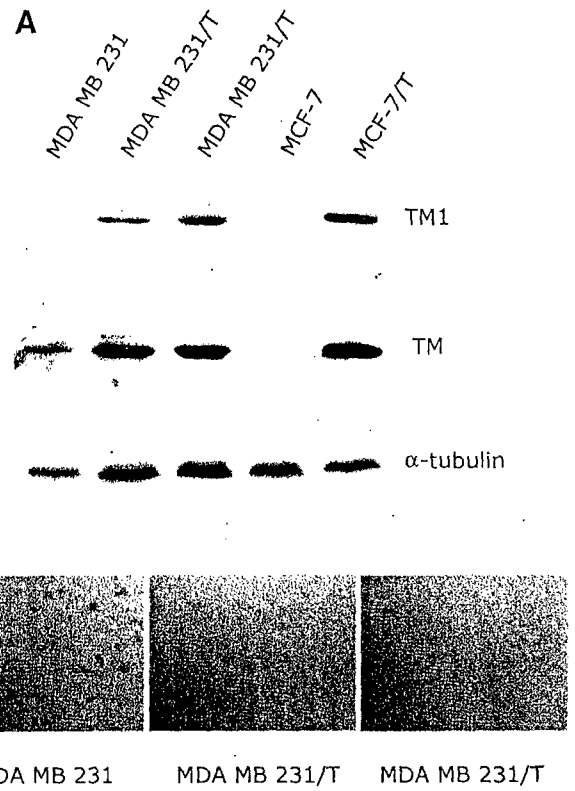

FIG. 8. TM1 expression suppresses the anchorage-independent growth of MDA MB 231 cells. (A) TM1 expression in MDA MB 231 cells and TM1-transduced cells was determined by immunoblotting with TM1-specific (top) and TM polyclonal (middle) antibodies, as described in Fig. 1B. (B) Ten thousand cells of MDA MB 231 and two MDA MB 231/T cells were cultured in soft agar for 3 weeks, and the colonies were enumerated as described under Materials and Methods. While MDA MB 231 cells grew in soft agar and formed 3660 colonies, with MDA MB 231/T cells no growth was detected.

E-cadherin or β-catenin, but results in the increased detergent solubility of these molecules [31]. Increased association with the cytoskeletal elements is also reflected in the localization of E-cadherin and β-catenin to cell–cell boundaries of the revertant cells. Current efforts are directed at investigating the biochemical basis of these interactions.

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REFERENCES

1. Kinzler, K. W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* **386,** 761, 763.
2. Sommers, C. L. (1996). The role of cadherin-mediated adhesion in breast cancer. *J. Mammary Gland Biol. Neoplasia* **1,** 219–229.
3. Hirohashi, S. (1998). Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am. J. Pathol.* **153,** 333–339.
4. Bissell, M. J., Weaver, V. M., Lelievre, S. A., Wang, F., Petersen, O. W., and Schmeichel, K. L. (1999). Tissue structure, nuclear organization, and gene expression in normal and malignant breast. *Cancer Res.* **59,** 1757–1763s; discussion 1763s–1764s.
5. Schwartz, M. A. (1997). Integrins, oncogenes, and anchorage independence. *J. Cell Biol.* **139,** 575–578.
6. Ishikawa, R., Yamashiro, S., and Matsumura, F. (1989). Annealing of gelsolin-severed actin fragments by tropomyosin in the presence of Ca2+. Potentiation of the annealing process by caldesmon. *J. Biol. Chem.* **264,** 16764–16770.
7. Ishikawa, R., Yamashiro, S., and Matsumura, F. (1989). Differential modulation of actin-severing activity of gelsolin by multiple isoforms of cultured rat cell tropomyosin. Potentiation of protective ability of tropomyosins by 83-kDa nonmuscle caldesmon. *J. Biol. Chem.* **264,** 7490–7497.
8. Blanchoin, L., Pollard, T. D., and Hitchcock-DeGregori, S. E. (2001). Inhibition of the Arp2/3 complex-nucleated actin polymerization and branch formation by tropomyosin. *Curr. Biol.* **11,** 1300–1304.
9. Wen, K. K., Kuang, B., and Rubenstein, P. A. (2000). Tropomyosin-dependent filament formation by a polymerization-defective mutant yeast actin (V266G,L267G). *J. Biol. Chem.* **275,** 40594–40600.
10. Strand, J., Nili, M., Homsher, E., and Tobacman, L. S. (2001). Modulation of myosin function by isoform-specific properties of *Saccharomyces cerevisiae* and muscle tropomyosins. *J. Biol. Chem.* **276,** 34832–34839.
11. Pruyne, D. W., Schott, D. H., and Bretscher, A. (1998). Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. *J. Cell Biol.* **143,** 1931–1945.
12. Bhattacharya, B., Prasad, G. L., Valverius, E. M., Salomon, D. S., and Cooper, H. L. (1990). Tropomyosins of human mammary epithelial cells: Consistent defects of expression in mammary carcinoma cell lines. *Cancer Res.* **50,** 2105–2112.
13. Matsumura, F., Lin, J. J., Yamashiro-Matsumura, S., Thomas, G. P., and Topp, W. C. (1983). Differential expression of tropomyosin forms in the microfilaments isolated from normal and transformed rat cultured cells. *J. Biol. Chem.* **258,** 13954–13964.
14. Cooper, H. L., Feuerstein, N., Noda, M., and Bassin, R. H. (1985). Suppression of tropomyosin synthesis, a common biochemical feature of oncogenesis by structurally diverse retroviral oncogenes. *Mol. Cell. Biol.* **5,** 972–983.
15. Cooper, H. L., Bhattacharya, B., Bassin, R. H., and Salomon, D. S. (1987). Suppression of synthesis and utilization of tropomyosin in mouse and rat fibroblasts by transforming growth factor alpha: A pathway in oncogene action. *Cancer Res.* **47,** 4493–4500.
16. Lin, C. S., and Leavitt, J. (1988). Cloning and characterization of a cDNA encoding transformation-sensitive tropomyosin isoform 3 from tumorigenic human fibroblasts. *Mol. Cell. Biol.* **8,** 160–168.
17. Lin, J. J., Warren, K. S., Wamboldt, D. D., Wang, T., and Lin, J. L. (1997). Tropomyosin isoforms in nonmuscle cells. *Int. Rev. Cytol.* **170,** 1–38.
18. Pawlak, G., and Helfman, D. M. (2001). Cytoskeletal changes in cell transformation and tumorigenesis. *Curr. Opin. Genet. Dev.* **11,** 41–47.
19. Prasad, G. L., Fuldner, R. A., and Cooper, H. L. (1993). Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene. *Proc. Natl. Acad. Sci. USA* **90,** 7039–7043.

20. Braverman, R. H., Cooper, H. L., Lee, H. S., and Prasad, G. L. (1996). Anti-oncogenic effects of tropomyosin: Isoform specificity and importance of protein coding sequences. *Oncogene* **13,** 537–545.
21. Prasad, G. L., Masuelli, L., Raj, M. H., and Harindranath, N. (1999). Suppression of src-induced transformed phenotype by expression of tropomyosin-1. *Oncogene* **18,** 2027–2031.
22. Pittenger, M. F., Kazzaz, J. A., and Helfman, D. M. (1994). Functional properties of non-muscle tropomyosin isoforms. *Curr. Opin. Cell Biol.* **6,** 96–104.
23. Ishikawa, R., Yamashiro, S., Kohama, K., and Matsumura, F. (1998). Regulation of actin binding and actin bundling activities of fascin by caldesmon coupled with tropomyosin. *J. Biol. Chem.* **273,** 26991–26997.
24. Yamashiro, S., Yamakita, Y., Ono, S., and Matsumura, F. (1998). Fascin, an actin-bundling protein, induces membrane protrusions and increases cell motility of epithelial cells. *Mol. Biol Cell* **9,** 993–1006.
25. Oldham, S. M., Clark, G. J., Gangarosa, L. M., Coffey, R. J., Jr., and Der, C. J. (1996). Activation of the Raf-1/MAP kinase cascade is not sufficient for Ras transformation of RIE-1 epithelial cells. *Proc. Natl. Acad. Sci. USA* **93,** 6924–6928.
26. Prasad, G. L., Valverius, E. M., McDuffie, E., and Cooper, H. L. (1992). cDNA cloning and expression of an epithelial cell protein, HME1, that is down regulated in neoplastic mammary cells. *Cell Growth. Differ.* **3,** 507–513.
27. Prasad, G. L., Meissner, P. S., Sheer, D., and Cooper, H. L. (1991). A cDNA encoding a muscle-type tropomyosin cloned from a human epithelial cell line: Identity with human fibroblast tropomyosin, TM1. *Biochem. Biophys. Res. Commun.* **177,** 1068–1075.
28. Shah, V., Braverman, R., and Prasad, G. L. (1998). Suppression of Neoplastic Transformation and Regulation of Cytoskeleton by Tropomyosins. *Somatic Cell Mol. Genet.* **24,** 273–280.
29. Shah, V., Bharadwaj, S., Kaibuchi, K., and Prasad, G. L. (2001). Cytoskeletal organization in tropomyosin-mediated reversion of ras-transformation: Evidence for Rho kinase pathway. *Oncogene* **20,** 2112–2121.
30. Prasad, G. L., Fuldner, R. A., Braverman, R., McDuffie, E., and Cooper, H. L. (1994). Expression, cytoskeletal utilization and dimer formation of tropomyosin derived from retroviral-mediated cDNA transfer. Metabolism of tropomyosin from transduced cDNA. *Eur. J. Biochem.* **224,** 1–10.
31. Kinch, M. S., Clark, G. J., Der, C. J., and Burridge, K. (1995). Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. *J. Cell Biol.* **130,** 461–471.
32. Kamarainen, M., Seppala, M., Virtanen, I., and Andersson, L. C. (1997). Expression of glycodelin in MCF-7 breast cancer cells induces differentiation into organized acinar epithelium. *Lab. Invest.* **77,** 565–573.
33. Gumbiner, B. M. (1997). Carcinogenesis: A balance between beta-catenin and APC. *Curr. Biol.* **7,** R443–R446.
34. Yap, A. S., Brieher, W. M., and Gumbiner, B. M. (1997). Molecular and functional analysis of cadherin-based adherens junctions. *Annu. Rev. Cell Dev. Biol.* **13,** 119–146.
35. Polakis, P., Hart, M., and Rubinfeld, B. (1999). Defects in the regulation of beta-catenin in colorectal cancer. *Adv. Exp. Med. Biol.* **470,** 23–32.
36. Polakis, P. (1999). The oncogenic activation of beta-catenin. *Curr. Opin. Genet. Dev.* **9,** 15–21.
37. Sommers, C. L., Byers, S. W., Thompson, E. W., Torri, J. A., and Gelmann, E. P. (1994). Differentiation state and invasiveness of human breast cancer cell lines. *Breast Cancer Res. Treat.* **31,** 325–335.
38. McCartney, B. M., Dierick, H. A., Kirkpatrick, C., Moline, M. M., Baas, A., Peifer, M., and Bejsovec, A. (1999). Drosophila APC2 is a cytoskeletally-associated protein that regulates wingless signaling in the embryonic epidermis. *J. Cell Biol.* **146,** 1303–1318.
39. McCartney, B. M., and Peifer, M. (2000). Teaching tumour suppressors new tricks [news]. *Nature Cell Biol.* **2,** E58–E60.
40. Bharadwaj, S., and Prasad, G. L. (2002). Tropomyosin-1, a novel suppressor of cellular transformation is downregulated by promoter methylation in cancer cells. *Cancer Lett.* **183,** 205–213.
41. Asgeirsson, K. S., JG, J. O., Tryggvadottir, L., Olafsdottir, K., Sigurgeirsdottir, J. R., Ingvarsson, S., and Ogmundsdottir, H. M. (2000). Altered expression of E-cadherin in breast cancer. patterns, mechanisms and clinical significance. *Eur J. Cancer* **36,** 1098–1106.
42. Bukholm, I. K., Nesland, J. M., Karesen, R., Jacobsen, U., and Borresen-Dale, A. L. (1998). E-cadherin and alpha-, beta-, and gamma-catenin protein expression in relation to metastasis in human breast carcinoma. *J. Pathol.* **185,** 262–266.
43. Bukholm, I. K., Nesland, J. M., and Borresen-Dale, A. L. (2000). Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients [seecomments]. *J. Pathol.* **190,** 15–19.
44. Mareel, M., Boterberg, T., Noe, V., Van Hoorde, L., Vermeulen, S., Bruyneel, E., and Bracke, M. (1997). E-cadherin/catenin/cytoskeleton complex: a regulator of cancer invasion. *J. Cell Phys.* **173,** 271–274.
45. Hagios, C., Lochter, A., and Bissell, M. J. (1998). Tissue architecture: the ultimate regulator of epithelial function? *Philos. Trans. R. Soc. London Ser. B. Biol.* **353,** 857–870.
46. Huiping, C., Sigurgeirsdottir, J. R., Jonasson, J. G., Eiriksdottir, G., Johannsdottir, J. T., Egilsson, V., and Ingvarsson, S. (1999). Chromosome alterations and E-cadherin gene mutations in human lobular breast cancer. *Br. J. Cancer* **81,** 1103–1110.

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APPENDIX 4

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Loss of expression of tropomyosin-1, a novel class II tumor suppressor that induces anoikis, in primary breast tumors

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Suppression of tropomyosins (TMs), a family of actin-binding, microfilament-associated proteins, is a prominent feature of many transformed cells. Yet it is unclear whether downregulation of TMs occur in human tumors. We have investigated the expression of tropomyosin-1 (TM1) in human breast carcinoma tissues by *in situ* hybridization and immunofluorescence. TM1 mRNA and protein are readily detectable in normal mammary tissue. In contrast, TM1 expression is abolished in the primary human breast tumors. Expression of other TM isoforms, however, is variable among the tumors. The consistent and profound downregulation of TM1 suggests that TM1 may be a novel and useful biomarker of mammary neoplasms. These data also support the hypothesis that suppression of TM1 expression during the malignant conversion of mammary epithelium as a contributing factor of breast cancer. In support of this hypothesis, we show that the ability to suppress malignant growth properties of breast cancer cells is specific to TM1 isoform. Investigations into the mechanisms of TM1-induced tumor suppression reveal that TM1 induces anoikis (detachment induced apoptosis) in breast cancer cells. Downregulation of TM1 in breast tumors may destabilize microfilament architecture and confer resistance to anoikis, which facilitates survival of neoplastic cells outside the normal microenvironment and promote malignant growth.
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Keywords: tropomyosin; breast cancer; tumor suppressor; anoikis; biomarker

Introduction

During neoplastic transformation, cells undergo numerous biochemical changes, some of which confer a selective advantage over their normal counterparts to facilitate malignant growth. These changes include mutational inactivation of tumor suppressor proteins, activation of oncogenes and other epigenetic events (Fearon and Vogelstein, 1990; Beckmann *et al.*, 1997). Elucidation of these causal molecular events is necessary to develop better tumor markers and identify effective targets for cancer treatment. A number of research groups have demonstrated that suppression of high-M_r tropomyosin (TM) isoforms (tropomyosin-1 and tropomyosin-2) occurs in malignant cells, suggesting a role for these proteins in neoplastic transformation (Hendricks and Weintraub, 1981; Matsumura *et al.*, 1983; Cooper *et al.*, 1985; Bhattacharya *et al.*, 1990).

TMs are a family of actin-binding proteins that stabilize microfilaments and are expressed with a high degree of tissue specificity (Lin *et al.*, 1997; Pawlak and Helfman, 2001). TMs may be broadly categorized into high- and low-M_r species depending on their size. In nonmuscle cells, such as fibroblasts and epithelial cells, multiple high-M_r TMs are expressed, which are referred to as isoforms TM1, TM2 and TM3; additionally, epithelial cells also express another protein designated as TM38. Similarly, smooth muscle cells express two TM isoforms, TM1 and TM2. Previous work from this laboratory has demonstrated that multiple TM isoforms are downregulated in human breast carcinoma cells (Bhattacharya *et al.*, 1990). More significantly, expression of TM1 was consistently abolished in most commonly studied breast cancer cell lines, indicating that loss of TM1 could be an important event in mammary carcinogenesis. This finding is in line with the general observations that most neoplastic cells exhibit altered cellular morphology and contain disorganized microfilaments, and that suppression of TM1 is a common biochemical event in cells transformed by diverse oncogenic modalities (Cooper *et al.*, 1985). We hypothesized that loss of TM1 results in the formation of disorganized microfilaments, which in turn, facilitate neoplastic conversion. Consistent with this hypothesis, TM1 reorganizes actin filaments and functions as a supp-ressor of malignant transformation (Prasad *et al.*, 1993, 1999; Braverman *et al.*, 1996; Mahadev *et al.*, 2002).

Since many breast carcinoma cell lines lack TM1, we considered whether TM1 could serve as a potential tumor marker. Two previous studies, utilizing biochemical methods, have suggested that TM1 expression may be enhanced in breast tumors (Franzen *et al.*,

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1996, 1997). However, we believe that accurate assessment of TM1 expression in tissues by biochemical methods is difficult for several reasons. For example, (1) multiple TM proteins share extensive sequence homology; (2) TM1 is expressed in multiple cell types present in tissues. In addition to epithelial and stromal cells, smooth muscle cells of tumor vasculature express abundant amounts of TM1 and; (3) the lack of isoform-specific antibodies has rendered the accurate assessment of TM1 protein by immunohistochemistry in human tissues difficult. In this communication, we have investigated whether TM1 expression is altered in human breast carcinomas, and the isoform specific breast cancer suppression by TMs. We also report that inhibition of malignant growth of breast cancer cells by TM1 involves induction of anoikis.

Results

Since TM1 expression is consistently lost in breast cancer cells, and TM1 suppresses the malignant growth of breast cancer cells (Mahadev *et al.*, 2002), we have chosen to investigate changes in TM1 expression in breast cancer. We utilized *in situ* hybridization and immunofluorescence methods to validate the RNA probes and TM1-specific antibody (data not shown) using MCF10A and MCF-7 cells, which contain known TM profiles (Bhattacharya *et al.*, 1990). TM1 expression was detected in MCF-10A cells; MCF-7 cells, on the other hand, lacked TM1 mRNA and protein, in agreement with the Northern blotting data and two-dimensional gel analyses.

TM1 mRNA expression is downregulated in breast cancer

To determine whether TM1 expression is altered in breast tumors, *in situ* hybridization was performed. Adjacent normal tissues from the breast cancer patients, as judged by gross examination and histology, were also used. TM1 expression was readily detectable by the intense labeling of silver grains in the normal ducts (Figure 1). Adjacent sections hybridized to the control sense probe lacked specific signal and pattern, and was diffuse and weak.

Analysis of the adjacent invasive tumor tissue revealed profound differences in TM1 expression compared to the normal tissue. The invasive breast tumor tissue lacked any detectable TM1 mRNA, and the signal obtained with the antisense probe and the control sense probe was essentially identical. The signal obtained with the antisense probe in tumor tissue was comparable to that obtained with the background signal. Analysis of five different normal ducts and malignant cells revealed significant differences between the tissues. The mean intensity, indicative of the abundance of TM1 mRNA in normal ducts, was 29.7 ± 8.1 (mean $\pm$ s.d.) after subtracting the background signal obtained with the sense probe. In contrast, TM1 mRNA levels in the tumor tissue corresponded to a mean value of 3.8 ± 2.8 over the background. Thus, TM1 expression is profoundly suppressed in breast tumors.

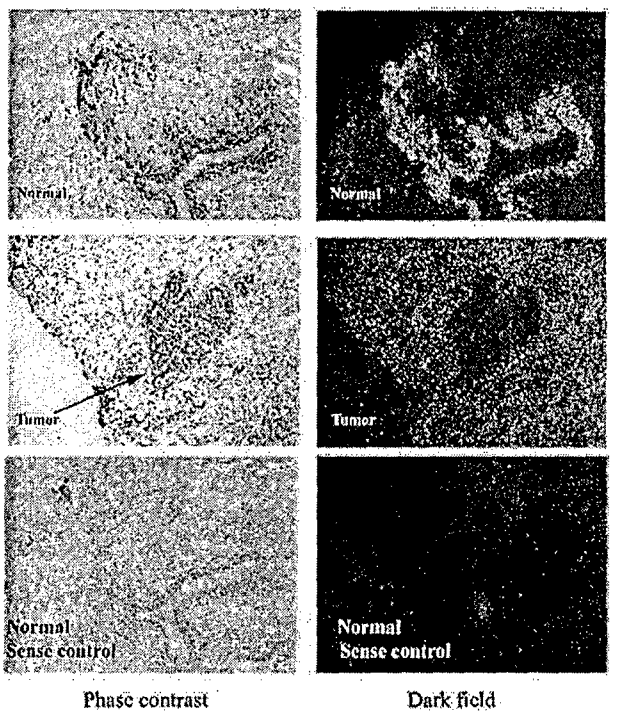

Figure 1 TM1 mRNA expression in normal and malignant breast tissue: normal and malignant breast tissues were hybridized with antisense (top and middle panels) and sense TM1 probes (bottom panel), and developed as described in Materials and methods. Samples were photographed in bright field to view the tissue architecture, and in dark field to view the silver grains indicative of TM1 expression. H&E staining of the tissue, sense probe hybridization of tumor tissue are not depicted. Samples were photographed at $\times 25$ magnification

Immunohistochemical analysis of TM1 expression in invasive breast cancer

To further evaluate changes in TM1 expression, we have performed immunohistochemistry on the same set of tissues used for *in situ* studies.

Immunofluorescence analysis of normal breast epithelium with a TM1-specific antibody (Mahadev *et al.*, 2002) revealed specific staining of both myoepithelial and luminal epithelial layers of the ducts for TM1 (Figure 2a). Omission of the primary antibody in the reaction (background) did not produce any detectable staining. Autofluorescence in the FITC channel revealed background signal that did not interfere with the TM1 signal.

Malignant breast epithelium, on the other hand, lacked any detectable TM1, and the signal intensity in the tumor area was equal to that obtained with omission of the primary antibody (Figure 2b). The blood vessels present in the tumor, however, stained intensely with TM1 antibody, indicating that the lack of staining in tumor tissue was not due to the inability of the antibody

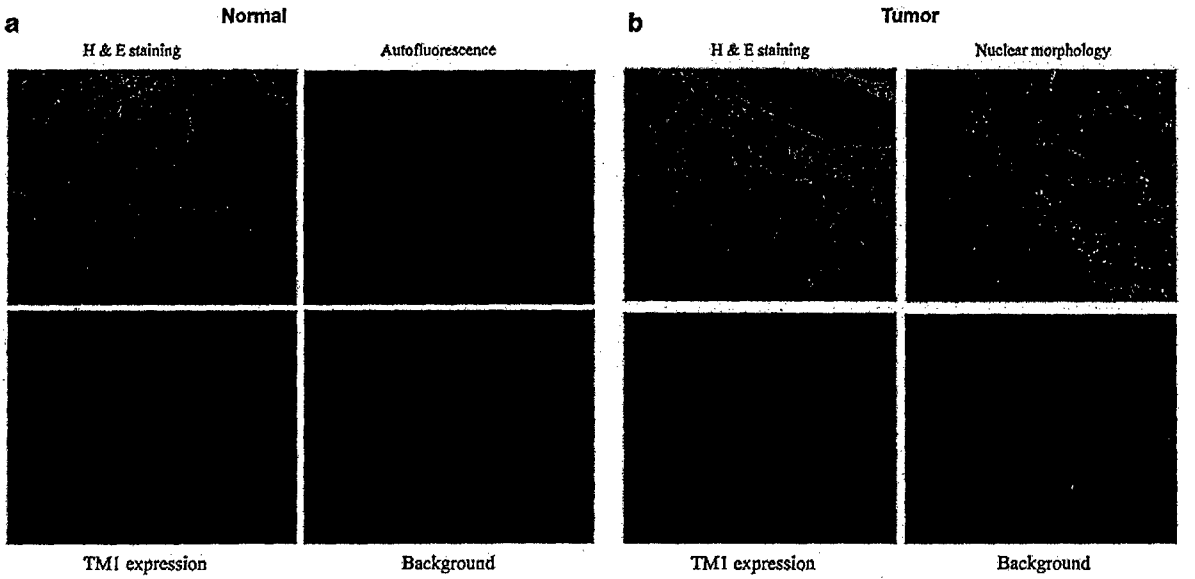

Figure 2 Expression of TM1 protein in normal and malignant breast tissues: (**a**) TM1 is expressed in normal ducts. Tissue sections were stained with TM1-specific antibody, and TM1 staining was viewed with a rhodamine cube, or autofluorescence was recorded through FITC cube. A parallel section was processed by omitting the primary antibody for negative control (background). H&E staining of the tissue sample is also shown. (**b**) Tumor tissue lacks expression of TM1: breast tumor stained with TM1-specific antibody (TM1 expression), negative control (background) and nuclear morphology along with H&E staining are shown. While the tumor area lacks TM1 staining, a blood vessel in the tumor reacts strongly, serving as a positive control for TM1 staining. × 25 magnification

to detect TM1. TM1 expression was undetectable in any of the 25 tumors tested, independent of any other parameters such as the stage, nodal status, hormone receptor status, proliferative index or HER2/neu status.

Quantitation of TM1 expression in tumor tissues revealed a significant decrease compared with the normal tissue. We measured relative luminosity in 10 normal and nine tumor tissue images, along with 14 background images taken from different tissues. Based on results from a mixed model, the adjusted mean levels of luminosity in normal tissues are 47.5 ± 1.1, for tumor tissues 16.2 ± 1.1, and the background signal is 15.3 ± 1.1 (mean $\pm$ s.e.). TM1 expression levels in normal tissues over the background are highly significant ($P < 0.0001$). The difference in TM1 protein levels between normal and tumor tissues is highly significant ($P < 0.0001$), indicating a profound downregulation of TM1 in breast tumors. However, the difference between tumor and the background signal was not significant ($P = 0.63$). These results demonstrate that while normal mammary tissues express abundant quantities of TM1, the malignant breast tumors essentially lack TM1.

To investigate, whether in addition to TM1, other TMs are also downregulated in breast tumors, tissue sections were screened with a pan-TM antibody which reacts preferentially with the high-M_r TMs, including TM1 (Bhattacharya *et al.*, 1990; Mahadev *et al.*, 2002). The normal ducts were intensely stained with the pan-TM antibody, indicating the expression of TM1 and other TMs in both the basal and luminal epithelial cells (Figure 3a). In breast tumor specimens, however, the staining pattern was variable. In most tumors (19/25, 76%) no signal was detected, indicating lack of or low level of expression of TMs (Figure 3b; Table 1). Blood vessels present in tumor tissue intensely stained, demonstrating the reactivity of TM antibody.

In a significant number (6/25 samples; 24% of breast tumors; Table 1) of tumor tissues, the pan-TM antibody reactivity was readily evident by the intense staining of tumor tissues, indicating the expression of other TM proteins (Figure 3c). The same set of samples stained negative for TM1 expression in parallel experiments, indicating that TM1 was consistently abolished in breast tumors, and expression of other TMs was variable. The widespread suppression of TM1 in breast carcinomas and the ability of TM1 to inhibit malignant growth of breast cancer cells strengthens the hypothesis that TM1 is a tumor suppressor (Prasad *et al.*, 1993, #5; Prasad *et al.*, 1999, #6; Mahadev *et al.*, 2002, #41). Taken together, our findings suggest that the loss of TM1 expression is a crucial event that directly contributes to mammary carcinogenesis.

Isoform-specific suppression of malignant growth of breast cancer cells by TM1

Since both TM1 and TM2 share extensive sequence homology (Figure 4a), and contrary to our findings (Braverman *et al.*, 1996), TM2 is suggested to function as a ras-suppressor (Janssen and Mier, 1997), we have tested the effect of restoration of TM2 in breast cancer cells. Stable expression of TM2 failed to inhibit the anchorage-independent growth of MCF-7 cells (Figure 4b). Both parental MCF-7 cells and those

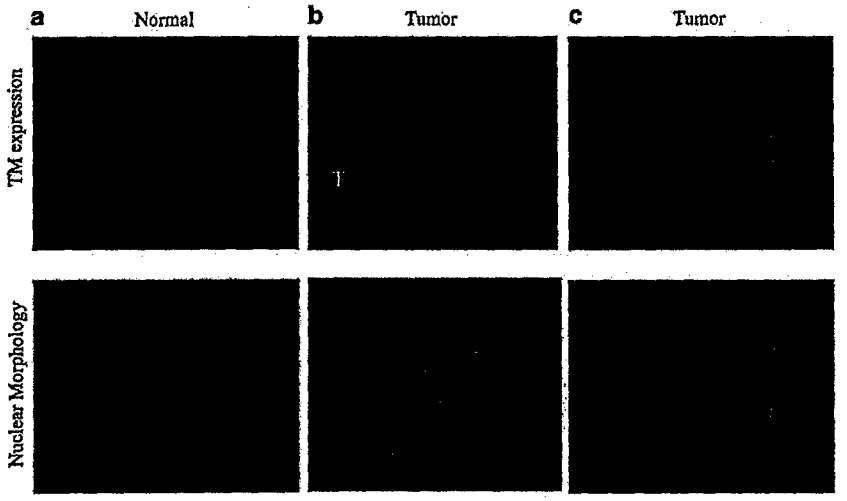

Figure 3 Expression of other TM isoforms is variable in breast tumors. Tissues were stained with a pan-TM antibody that reacts with multiple TM proteins (top panels, labeled as TM expression). The samples were also reacted with DAPI to visualize nuclear morphology and tissue architecture (bottom panels, marked as Nuclear morphology) following the antibody incubations. (**a**) The pan-TM antibody detects TMs isoforms in mammary epithelium. (**b**) A tumor sample with no detectable TM staining. However, TMs expressed in a blood vessel are readily detected. Area of the tumor is identified with 'T' in the top panel. (**c**) A tumor sample with reactivity to pan-TM antibody

Table 1 TM expression in breast tumors

Tissues	*Number of tissues expressing*	
	TM1	*Other TMs*
Normal breast epithelium	All	All
Malignant breast tissue	0/25	6/25

expressing TM2 formed colonies in agar, indicating that TM2 is not a suppressor of transformation in breast cancer cells.

Adenoviral transduction of TM1, on the other hand, suppresses anchorage-independent growth of MCF-7 cells (Figure 4c). We have transduced MCF-7 cells using the AdEasy system, which allows for coexpression of green fluorescent protein (GFP) along with wild-type TM1 (AdTM1). TM1 expressing MCF-7 cells, visualized by GFP expression, failed to grow under anchorage-independent conditions, while those not infected with AdTM1 virus, or those transduced with the control virus expressing only GFP (AdGFP), grew aggressively. Since MCF-7 cells grow as clumps, during the initial culture period, GFP expressing clumps were evident, which diminished with time. At the end of 20 days of culture, essentially all of the TM1 expressing cells died, as observed by lack of GFP expressing cells. MCF-7 cells transduced with AdGFP virus yielded 4870 colonies of cells positive for GFP in this representative experiment. In contrast, transduction of MCF-7 cells with TM1 resulted in a 50% decrease in total number of colonies compared to those infected with the control virus, reflecting the 50% efficiency of the adenoviral transduction in this experiment. These data further support the isoform specificity of TM1-mediated suppression of the malignant growth properties of breast cancer cells (Braverman *et al.*, 1996).

TM1 induces anoikis in breast cancer cells

In efforts to better understand TM1-mediated tumor suppression, we have investigated whether TM1 modulates cell cycle. As depicted in Figure 5a, asynchronous populations of MCF-7/TM1 cells contained significantly lower fraction of cells in S phase compared to MCF-7 cells. The number of cells in G_2–M phase was slightly higher in MCF-7/TM1 cells than in the parental cells. Furthermore, 5-bromo-2′-deoxyuridine (BrdU) incorporation studies (Figure 5b) also revealed that TM1 expression decreases the S phase in MCF-7 cells. The S-phase fraction values for MCF-7 cells (34.4%) were significantly higher than with MCF-7/TM1 (20.7%), indicating a lower rate of DNA synthesis. Interestingly, a significant number of MCF-7/TM1 cells, although were in S phase (based on DNA content), remained 'inactive' as judged by BrdU incorporation (Figure 5b). Thus, the slower monolayer growth of MCF-7/TM1 cells observed in previous studies (Mahadev *et al.*, 2002) is due to a decrease in S-phase fraction, and does not fully explain tumor suppression by TM1.

One of the hallmarks of malignant growth is to acquire resistance to undergo apoptosis when deprived of normal cell–matrix interactions, also known as anoikis (Frisch and Screaton, 2001; Stupack and Cheresh, 2002). TM1 restoration in many transformed cells profoundly suppresses anchorage-independent growth, indicating that TM1 resensitizes malignant cells to adhesion-dependent survival. Therefore, we have further investigated whether TM1-mediated tumor suppression involves anoikis.

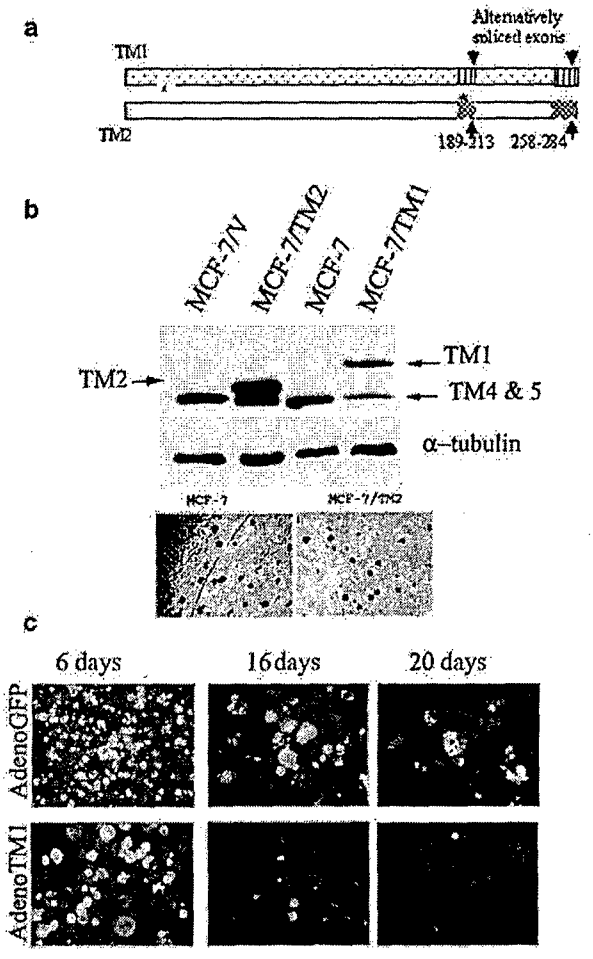

Figure 4 Isoform specificity of tumor suppression by TM1. (a) Comparison of TM1 and TM2 proteins. TM1 and TM2 share extensive sequence homology except in the sequence coded by two alternatively spliced exons (amino acids 189–213 and 258–284). The asterisks identify unique cysteine residues in TM1 (cys 35) and TM2 (cys 190). (b) TM2 is not a tumor suppressor of breast cancer cells: MCF-7 cells were transfected to restore the expression of TM2, and stably selected cells were tested for TM2 expression using a commercial antibody (TM311, Sigma). Unmodified MCF-7 cells, empty vector transduced cells (MCF-7/V), TM2 and TM1 expressing MCF-7 cells were used in the immunoblotting experiment (top). Parental and TM2 expressing MCF-7 cells were cultured under anchorage-independent conditions (bottom). (c) Adenoviral transduction of TM1 suppresses anchorage-independent growth of MCF-7 cells: control and TM1 expressing adenoviruses were used to infect MCF-7 cells at 50% efficiency for 24 h, and then 2×10^4 cells were plated on soft agar. Colony formation was monitored and the GFP-expression was recorded. TM1 expressing cells did not grow, as evidenced by lack of GFP signal, while the control vector transduction yielded a large number of GFP-positive colonies. Images were captured at × 10 magnification using FITC cube. The bright field images are not shown. Results of a representative experiment are shown

MCF-7/TM1 and MDA MB 231/TM1 were cultured in suspension and tested for cell survival and apoptosis. Unmodified MCF-7 cells, and those transduced with TM1 were cultured on polyHEMA-coated dishes to prevent adhesion to substratum, and examined nuclear morphology to determine whether TM1 induces anoikis. MCF-7/TM1 cells, when cultured in suspension, exhibited fragmented and dense nuclei consistent with apoptosis, indicating that TM1 induces anoikis in MCF-7 cells (Figure 6a). However, there was no evidence of apoptosis in parallel suspension cultures of MCF-7 cells (Figure 6a), or adherent cultures of MCF-7 and MCF-7/TM1 cells (data not shown). Next, we investigated whether MCF-7/TM2 cells undergo anoikis. Unlike, MCF-7/TM1 cells, MCF-7/TM2 cells had exhibited resistance to anoikis, as determined by nuclear morphology (Figure 6a). Consistent with this result, parental MCF-7 and MCF-7/TM2 cells did not accumulate DNA in subG_0–G_1 fraction of cell cycle, which is indicative of apoptosis, and the cell cycle pattern was comparable to that of the adherent cells (data not shown). These results indicate that TM1, but not TM2, sensitize breast cancer cells to detatchment-induced apoptosis, which is in agreement with the results of anchorage-independent growth experiments (Figure 4a).

To further examine whether TM1-mediated tumor suppression of breast cancer involves anoikis or it is a cell-type-specific effect in MCF-7 cells, we tested the ability of TM1 to sensitize MDA MB 231 cells to undergo detachment-induced apoptosis. As shown in Figure 6b, MDA MB 231/TM1 cells were undergoing massive cell death (70%) by 24 h of suspension culture, while unmodified MDA MB 231 cells exhibited background apoptosis (8.3%). A likely reason for rapid induction of anoikis by TM1 in MDA MB 231 cells is that MCF-7 cells exhibit stronger cell–cell adhesion due to the expression of E-cadherin (Sommers *et al.*, 1994). Since cell–cell interactions also provide survival signals, the onset of anoikis may have been delayed in MCF-7 cells expressing TM1. Moreover, TM1 expression appears to result in increased cytoskeletal association of E-cadherin complex (Mahadev *et al.*, 2002), presumably leading to enhanced cell–cell interactions.

Since TM1 expression induces profound and rapid anoikis in MDA MB 231 cells, we next examined the kinetics of induction of anoikis by TM1 by flow cytometry, and determined the accumulation of DNA in subG_0–G_1 fraction of cell cycle. By 6 h of culture in suspension, TM1 expressing cells accumulated 34% of DNA in subG_0-G_1 fraction, (Figure 6b and c). In contrast, MDA MB 231 cells did not contain any DNA in the subG_0–G_1 fraction, indicating normal growth (Figure 6a). The subG_0–G_1 phase increased with time in suspension cultures of MDA MB 231/TM1 cells reaching 70%. MDA MB 231 cells, in contrast, exhibited normal cell cycle pattern and significantly lower (about 13%) DNA in subG_0–G_1 fraction compared to TM1 expressing cells by 24 h (Figure 6c). Adherent cultures of both cell types, however, contained background ($\leq$5%) quantities of fragmented DNA (boxes marked 'adh', Figure 6c). These data indicate that TM1 induces anoikis in breast cancer cells, and resensitization of the tumor cells to anoikis is an important mechanism through which TM1 exerts tumor suppression.

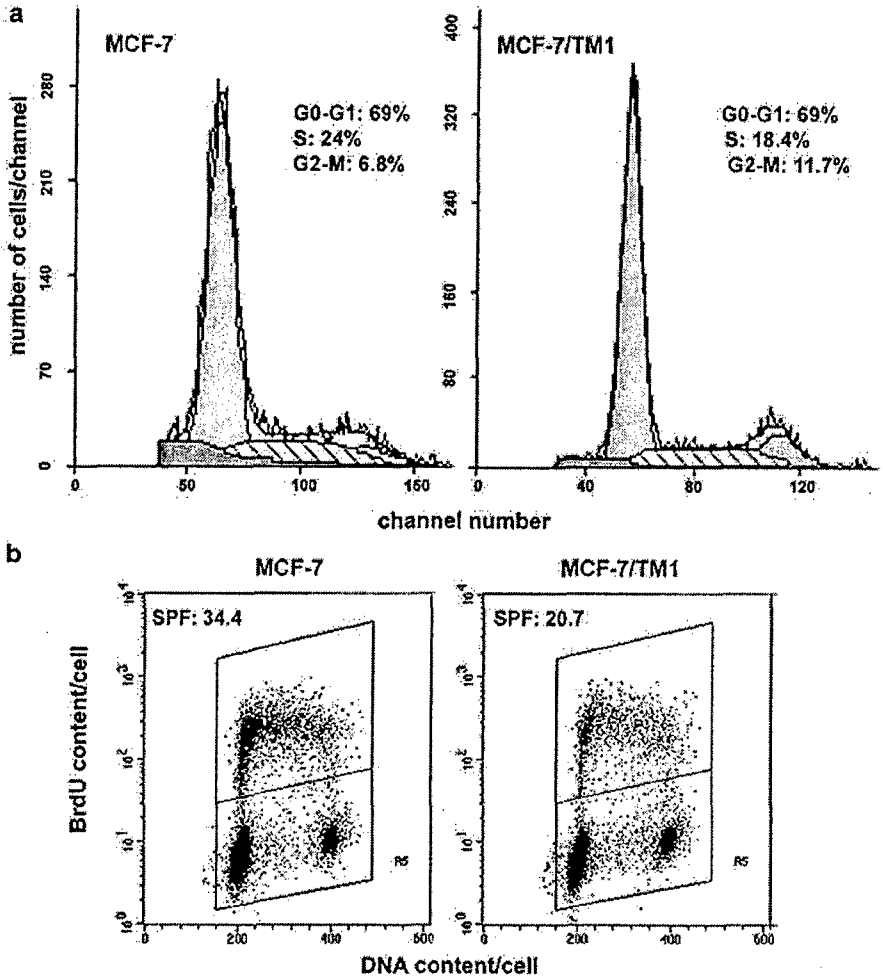

Figure 5 TM1-induced cell cycle changes MCF-7 cells. (**a**) Asynchronously growing MCF-7 and MCF-7/TM1 cells were subjected to cell cycle analysis. Trypsinized cells were detergent extracted, stained with propidium iodide and analysed by flow cytometry. Distribution of cells in each phase of the cells is given. (**b**) S-phase analysis was carried out by pulse labeling cells with BrdU as described in Materials and methods. The S-phase fraction values (SPF), indicative of the synthesis were calculated from two independent experiments. The average SPF values for MCF-7/TM1 were derived from experiments involving three independent clones

Discussion

The present work demonstrates that downregulation of TM1 expression occurs in invasive breast cancers, and that restoration of TM1 expression induces anoikis in breast cancer cells. Loss of TM1 expression is likely to destabilize microfilament architecture, and render breast tumor cells resistant to anoikis. These changes may facilitate, invasion and increased survival of tumor cells as they leave their primary locations and become malignant. Thus, suppression of TM1 may promote cytoskeletal disorganization and confer a growth advantage to the neoplastic cells.

The high-M_r TMs, including TM1, are abundantly expressed in the smooth muscle cells of the vasculature (Figures 2b and 3b) (Pittenger *et al.*, 1994). Furthermore, TM1 is expressed in fibroblasts, smooth muscle and epithelial cells (Prasad *et al.*, 1991; Pittenger *et al.*, 1994), which complicates accurate quantification of TM1 in tissues by biochemical methods. Accordingly, other researchers have reported a modest decrease in high-M_r TMs in primary breast tumors, and an increase in TM1 expression in the metastatic tumors (Franzen *et al.*, 1996, 1997). Similarly, a preliminary report indicates variable expression of TMs in prostate tumors (Ahram *et al.*, 2002). Therefore, we employed *in situ* hybridization and isoform-specific antibodies in immunohistochemistry, and have demonstrated that TM1 expression is downregulated in breast tumors.

TM1 expression, however, appears to be more variable in other common malignancies such as colon and lung cancer cell lines (data not shown). It is relevant to note that enhanced expression of either TM1, or other TMs in ras-transformed RIE cells has no effect on either cell growth or cytoskeletal organization (Shields *et al.*, 2002). Therefore, it appears that TM1 effects may

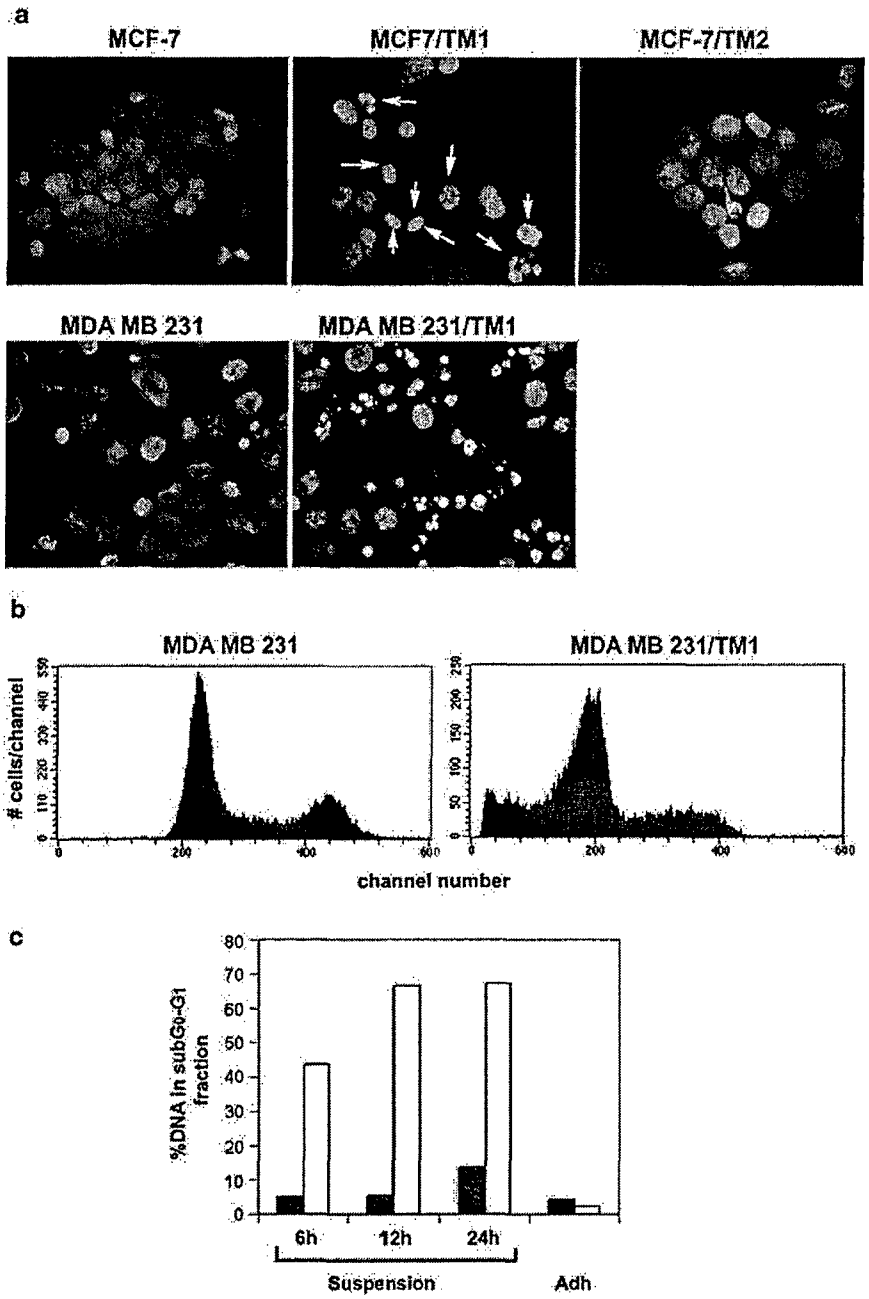

Figure 6 TM1 induces anoikis in breast cancer cells: parental MCF-7 and MDA MB 231 cells, and those expressing TM1 and TM2 were cultured on polyHEMA-coated dishes for 72 h (MCF-7-derived cells) or 24 h (MDA MB 231 derived cells). (**a**) Nuclear morphology of DAPI-stained cells is shown. Apoptotic cells are marked with arrows in MCF-7/TM1 cell panel. Cells were photographed using a fluorescent microscope with a UV filter cube at ×100 magnification. (**b** and **c**) TM1 expression induces rapid anoikis: accumulation of DNA in sub G_0–G_1 fraction, which indicates apoptosis, was measured by propidium iodide staining. MDA MB 231 and MDA MB 231/TM1 expressing cells were cultured in suspension for 6–24 h and harvested. Cell cycle distribution at 6 h is shown in (**b**). The percent DNA content in subG_0–G_1 fraction is shown (**c**). Shaded boxes are MDA MB 231 cells and the open boxes are MDA MB 231/TM1 cells. DNA content in subG_0–G_1 fraction of cells cultured under normal adhesion conditions (marked 'adh') has been quantitated. These samples do not contain any significant amount of apoptotic DNA

be tissue specific, or the transformed RIE cells may have other defects downstream of TM1, rendering TM1 ineffective.

Downregulation of TM1 appears to be one of the most common and yet remarkable changes in breast cancer. In addition to TM1, other class II tumor suppressors such as NES1 (Dhar *et al.*, 2001) and HME1 (14-3-3σ) (Ferguson *et al.*, 2000; Umbricht *et al.*, 2001) are also profoundly downregulated in a majority of breast cancers. Another actin-binding protein,

gelsolin, which severs F-actin and involved in dynamic remodeling of actin filaments, is also widely suppressed in breast tumors (Asch *et al.*, 1996; Mielnicki *et al.*, 1999).

Gross rearrangements in TM1 gene in breast tumors have not been detected (data not shown). Recent experiments with cultured breast cancer cell lines reveal that TM1 is silenced by epigenetic mechanisms involving gene methylation and histone deacetylation, which suggest that epigenetic mechanisms may regulate TM1 expression in tumors (Bharadwaj and Prasad, 2002). Thus, along with retinoic acid receptor (Sirchia *et al.*, 2000), estrogen receptor and E-cadherin (Nass *et al.*, 2000), 14-3-3σ (Ferguson *et al.*, 2000) and NES1 (Li *et al.*, 2001), TM1 gene is also turned off epigenetically during malignant transformation. It is interesting to note that while mutations in αTMs cause familial hypertrophic cardiomyopathy (Michele and Metzger, 2000) and nemaline myopathy (Donner *et al.*, 2002), the epigenetic mechanisms (Yang *et al.*, 2001) contribute to the loss of β TM gene function in neoplastic cells. However, fusions between low-M_r TMs and protein kinases such as trk receptor (Mitra *et al.*, 1987; Coulier *et al.*, 1989) and anaplastic lymphoma kinase (ALK) (Meech *et al.*, 2001) have been reported to be associated with malignant transformation. A transformation-specific low-M_r TM isoform is reported to be expressed in colon tumors, but not in normal colonic epithelia (Lin *et al.*, 2002).

While changes in the expression and activities of several key focal adhesion proteins, such as FAK (Xu *et al.*, 2000), ILK (Persad *et al.*, 2000) and Src (Coll *et al.*, 2002; Windham *et al.*, 2002), are implicated in anoikis, resensitization of breast cancer cells to anoikis by TM1, a microfilament-associated protein, is novel. Thus, TM1, like other tumor suppressors such as PTEN (Lu *et al.*, 1999), DOC/hab (Wang *et al.*, 2001) induces anoikis. Further investigations are underway to elucidate the molecular mechanisms of TM1-induced anoikis.

Materials and methods

Cell lines and reagents

Culture conditions for untransformed MCF10A, and the transformed MCF-7 and MDA MB 231 cells were previously described (Prasad *et al.*, 1992). A full-length TM1 cDNA clone (Prasad *et al.*, 1991) was subcloned into pGEM3 vector (Promega) to generate 'antisense' and 'sense probes'. The RNA probes were labeled with [^{35}S]UTP by standard techniques for *in situ* hybridization. TM1 cDNA was cloned into pAdTrack CMV plasmid and recombined with the viral backbone pAdEasy-1 vector, and transfected into HEK293 cells (He *et al.*, 1998). TM2 (Braverman *et al.*, 1996) was subcloned into pIRES2-EGFP plasmid (Clontech), and MCF-7 cells were transfected with the recombinant plasmid to coexpress GFP via an IRES sequence located downstream of the cloned TM2 cDNA.

A polyclonal pan-TM antibody that recognizes multiple TM proteins including TM1 has been previously described (Bhattacharya *et al.*, 1990; Prasad *et al.*, 1993; Mahadev *et al.*, 2002). This antibody recognizes high-M_r TMs more avidly, although it reacts with low-M_r proteins, as well. To detect TM1 specifically, we have generated several antipeptide antibodies and have determined them to be specific for TM1 (Bharadwaj and Prasad, 2002; Mahadev *et al.*, 2002). We have used one of the antibodies in immunofluorescence analyses to determine TM1 expression.

Tissue specimens

Aliquots of normal breast and tumor samples were obtained from patients undergoing surgery for their disease, through the Tumor Bank of Wake Forest University School of Medicine, Winston-Salem, NC, under the approval of the Institutional Review Board. Tissue samples (via mastectomy or lumpectomy) were collected within 60 min of surgery, fixed for 48 h in 10% phosphate-buffered formalin (pH 7.4) at room temperature, washed in 70% ethanol, embedded in paraffin, and cut into 5 μm sections for *in situ* hybridization, H&E staining and immunostaining. We have analysed a total of 25 breast tumors and their characteristics are given in Table 2.

Tissue culture cells with known TM protein profiles were trypsinized and fixed in 4% paraformaldehyde and washed in 98% ethanol followed by a brief wash with a mixture of 98% ethanol and ether (1 : 1 v/v). The cell pellet was dried and embedded in paraffin. Sections (5 μm) were cut and used in *in situ* hybridization and immunofluorescence experiments. These sections were used to test and validate the screening methods.

In situ *hybridization*

To detect TM1 mRNA, *in situ* hybridization was performed using a method described by Wilcox *et al.* with some modifications (http://www.emory.edu/WILCOX). The formalin-fixed, paraffin-embedded tissue sections were deparafinized and hydrated, and processed for *in situ* hybridization as previously described (Mondy *et al.*, 1997). For quantitation of TM1 mRNA signal, nonoverlaping images were imported into an image analysis program essentially as described (Mondy *et al.*, 1997). Corresponding areas from parallel sections probed for nonspecific hybridization were subtracted. Results from each sample were averaged, and comparisons were made between the tumor and normal area.

Immunocytochemical analysis of TM expression in breast tumors

Deparaffinized tissue specimens were employed for immunofluorescence using TM1-specific antibody or pan-TM-antibody as the primary antibody. Serial 5-μm-thick tissue sections were subjected to immunofluorescence staining for detection of TM1, or multiple TMs as described previously (Shah *et al.*, 2001; Mahadev *et al.*, 2002). For control purposes, samples were processed in parallel except with the omission of the primary antibody. Anti-rabbit immunoglobulin antibody conjugated with Texas Red was used as the secondary

Table 2 Breast tissues used to analyse TM expression

Total tissues analysed—25
Age range 35–87years, median age 54 years
Race: 24 w/f; 2 b/f
Ductal carcinoma – 21
Lobular carcinoma – 2
Phyllodes tumor – 1
Not determined from the initial pathology reports (unknown) – 1
Adjacent normal tissue – 24

antibody (Molecular Probes) followed by counterstaining with 10 ng/ml DAPI (in methanol w/v) to visualize the nuclei and to identify the tissue architecture. Microscopic analysis was performed using a ×25 Neofluar oil objective. Microscopic fields were captured with a Zeiss Axiocam camera, keeping the gain and exposure constant. Autofluorescence of tissue sections was observed using green fluorescence (FITC) channel, which did not interfere with the specific signal. TM1 expression was determined by quantifying the luminosity of the images using Adobe Photoshop (version 6.0) with the magic wand tool and the histogram function.

Cell cycle and anoikis experiments

Cell cycle analyses using propidium iodide and BrdU were carried out as described (Darzynkiewicz *et al.*, 2001). Briefly, cells were labeled for 1 h with BrdU (Sigma) at 20 μM concentration, harvested and fixed. Cells were reacted with anti-BrdU antibody (Becton-Dickinson) followed by incubation with FITC-conjugated second antibody (Sigma). Finally, the samples were stained with 20 μg/ml propidium iodide and subjected to flow cytometric analysis. S-phase fraction was calculated as a percentage of Brdu containing cells in the total cells (combined fractions of propidium iodide and BrdU containing cells).

Anoikis experiments were carried out on polyHEMA (Sigma) coated dishes (final concentration 10 μg/ml, w/v in ethanol, two applications) as described by Zhu *et al.* (2001). Cells were cultured in serum-free, 1% BSA containing medium. Cells were harvested and collected by Cyospin, and stained with 10 ng/ml DAPI to visualize nuclei. The cells were photographed using a Zeiss Axioplan 2 microscope (Carl Zeiss, Germany) with a ×100 objective, and a Zeiss Axiocam camera. Images were imported into Adobe Photoshop. To calculate the percent cells undergoing anoikis, several image fields were photographed and the percent apoptotic nuclei were counted. The DNA content in subG_0–G_1 fraction was calculated as flow cytometry (Darzynkiewicz *et al.*, 2001).

Other methods

Northern blot analysis was used to quantify TM1 mRNA expression (Mahadev *et al.*, 2002). For protein analyses, cultured cells were solubilized in lysis buffer (Mahadev *et al.*, 2002). The membranes were probed with TM1-specific antibody or pan-TM antibody and α-tubulin antibody (Bharadwaj and Prasad, 2002; Mahadev *et al.*, 2002). Anchorage-independent growth was measured by plating 2×10^4 cells on soft agar plates and culturing for 2–3 weeks. In experiments involving adenoviral infections, cells were infected for 24 h and then plated on soft agar. To transduce TM1, the recombinant virus at 180 MOI was used and the control virus was used at 100 MOI to achieve about 50% infectivity of MCF-7 cells, as measured by GFP expression. Statistical analyses were performed using PROC MIXED within the Statistical Analysis System for personal computers (SAS Institute, Cary, NC, USA).

Acknowledgements
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References

Ahram M, Best CJ, Flaig MJ, Gillespie JW, Leiva IM, Chuaqui RF, Zhou G, Shu H, Duray PH, Linehan WM, Raffeld M, Ornstein DK, Zhao Y, Petricoin III EF and Emmert-Buck MR. (2002). *Mol. Carcinog.*, **33,** 9–15.

Asch HL, Head K, Dong Y, Natoli F, Winston JS, Connolly JL and Asch BB. (1996). *Cancer Res.*, **56,** 4841–4845.

Beckmann MW, Niederacher D, Schnurch HG, Gusterson BA and Bender HG. (1997). *J. Mol. Med.*, **75,** 429–439.

Bharadwaj S and Prasad GL. (2002). *Cancer Lett.*, **183,** 205–213.

Bhattacharya B, Prasad GL, Valverius EM, Salomon DS and Cooper HL. (1990). *Cancer Res.*, **50,** 2105–2112.

Braverman RH, Cooper HL, Lee HS and Prasad GL. (1996). *Oncogene*, **13,** 537–545.

Coll ML, Rosen K, Ladeda V and Filmus J. (2002). *Oncogene*, **21,** 2908–2913.

Cooper HL, Feuerstein N, Noda M and Bassin RH. (1985). *Mol. Cell. Biol.*, **5,** 972–983.

Coulier F, Martin-Zanca D, Ernst M and Barbacid M. (1989). *Mol. Apoptosis, Cell. Biol.*, **9,** 15–23.

Darzynkiewicz Z, KLi X and Bedner E. (2001). *Methods Cell Biol. Vol. 66.* Schawartz LM and Ashwell JD (eds). Academic Press: San Diego, pp. 69–109.

Dhar S, Bhargava R, Yunes M, Li B, Goyal J, Naber SP, Wazer DE and Band V. (2001). *Clin. Cancer Res.*, **7,** 3393–3398.

Donner K, Ollikainen M, Ridanpaa M, Christen HJ, Goebel HH, de Visser M, Pelin K and Wallgren-Pettersson C. (2002). *Neuromuscular Disorders*, **12,** 151–158.

Fearon ER and Vogelstein B. (1990). *Cell*, **61,** 759–767.

Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR and Sukumar S. (2000). *Proc. Natl. Acad. Sci. USA*, **97,** 6049–6054.

Franzen B, Linder S, Alaiya AA, Eriksson E, Fujioka K, Bergman AC, Jornvall H and Auer G. (1997). *Electrophoresis*, **18,** 582–587.

Franzen B, Linder S, Uryu K, Alaiya AA, Hirano T, Kato H and Auer G. (1996). *Br. J. Cancer*, **73,** 909–913.

Frisch SM and Screaton RA. (2001). *Curr. Opin. Cell Biol.*, **13,** 555–562.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW and Vogelstein B. (1998). *Proc. Natl. Acad. Sci. USA*, **95,** 2509–2514.

Hendricks M and Weintraub H. (1981). *Proc. Natl. Acad. Sci. USA*, **78,** 5633–5637.

Janssen RA and Mier JW. (1997). *Mol. Biol. Cell*, **8,** 897–908.

Li B, Goyal J, Dhar S, Dimri G, Evron E, Sukumar S, Wazer DE and Band V. (2001). *Cancer Res.*, **61,** 8014–8021.

Lin JJ, Warren KS, Wamboldt DD, Wang T and Lin JL. (1997). *Intl. Rev. Cytol.*, **170,** 1–38.

Lin JL, Geng X, Bhattacharya SD, Yu JR, Reiter RS, Sastri B, Glazier KD, Mirza ZK, Wang KK, Amenta PS, Das KM and Lin JJ. (2002). *Gastroenterology*, **123,** 152–162.

Lu Y, Lin YZ, LaPushin R, Cuevas B, Fang X, Yu SX, Davies MA, Khan H, Furui T, Mao M, Zinner R, Hung MC, Steck P, Siminovitch K and Mills GB. (1999). *Oncogene*, **18,** 7034–7045.

Mahadev K, Raval G, Bharadwaj S, Willingham MC, Lange EM, Vonderhaar B, Salomon D and Prasad GL. (2002). *Exp. Cell Res.*, **279**, 40–51.

Matsumura F, Lin JJ, Yamashiro-Matsumura S, Thomas GP and Topp WC. (1983). *J. Biol. Chem.*, **258**, 13954–13964.

Meech SJ, McGavran L, Odom LF, Liang X, Meltesen L, Gump J, Wei Q, Carlsen S and Hunger SP. (2001). *Blood*, **98**, 1209–1216.

Michele DE and Metzger JM. (2000). *J. Mol. Med.*, **78**, 543–553.

Mielnicki LM, Ying AM, Head KL, Asch HL and Asch BB. (1999). *Exp. Cell Res.*, **249**, 161–176.

Mitra G, Martin-Zanca D and Barbacid M. (1987). *Proc. Natl. Acad. Sci. USA*, **84**, 6707–6711.

Mondy JS, Lindner V, Miyashiro JK, Berk BC, Dean RH and Geary RL. (1997). *Circ. Res.*, **81**, 320–327.

Nass SJ, Herman JG, Gabrielson E, Iversen PW, Parl FF, Davidson NE and Graff JR. (2000). *Cancer Res.*, **60**, 4346–4348.

Pawlak G and Helfman DM. (2001). *Curr. Opin. Gen. Dev.*, **11**, 41–47.

Persad S, Attwell S, Gray V, Delcommenne M, Troussard A, Sanghera J and Dedhar S. (2000). *Proc. Natl. Acad. Sci. USA*, **97**, 3207–3212.

Pittenger MF, Kazzaz JA and Helfman DM. (1994). *Curr. Opin. Cell Biol.*, **6**, 96–104.

Prasad GL, Fuldner RA and Cooper HL. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 7039–7043.

Prasad GL, Masuelli L, Raj MH and Harindranath N. (1999). *Oncogene*, **18**, 2027–2031.

Prasad GL, Meissner PS, Sheer D and Cooper HL. (1991). *Biochem. Biophys. Res. Commun.*, **177**, 1068–1075.

Prasad GL, Valverius EM, McDuffie E and Cooper HL. (1992). *Cell Growth Differentiation*, **3**, 507–513.

Shah V, Bharadwaj S, Kaibuchi K and Prasad GL. (2001). *Oncogene*, **20**, 2112–2121.

Shields JM, Mehta H, Pruitt K and Der CJ. (2002). *Mol. Cell. Biol.*, **22**, 2304–2317.

Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S and Sacchi N. (2000). *Oncogene*, **19**, 1556–1563.

Sommers CL, Byers SW, Thompson EW, Torri JA and Gelmann EP. (1994). *Breast Cancer Res. Treat.*, **31**, 325–335.

Stupack DG and Cheresh DA. (2002). *J. Cell Sci.*, **115**, 3729–3738.

Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J and Sukumar S. (2001). *Oncogene*, **20**, 3348–3353.

Wang SC, Makino K, Xia W, Kim JS, Im SA, Peng H, Mok SC, Singletary SE and Hung MC. (2001). *Oncogene*, **20**, 6960–6964.

Windham TC, Parikh NU, Siwak DR, Summy JM, McConkey DJ, Kraker AJ and Gallick GE. (2002). *Oncogene*, **21**, 7797–7807.

Xu LH, Yang X, Bradham CA, Brenner DA, Baldwin Jr AS, Craven RJ and Cance WG. (2000). *J. Biol. Chem.*, **275**, 30597–30604.

Yang X, Yan L and Davidson NE. (2001). *Endocrine-Relat. Cancer*, **8**, 115–127.

Zhu Z, Sanchez-Sweatman O, Huang X, Wiltrout R, Khokha R, Zhao Q and Gorelik E. (2001). *Cancer Res.*, **61**, 1707–1716.

APPENDIX 5

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N Terminus Is Essential for Tropomyosin Functions

N-TERMINAL MODIFICATION DISRUPTS STRESS FIBER ORGANIZATION AND ABOLISHES ANTI-ONCOGENIC EFFECTS OF TROPOMYOSIN-1*

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Down-regulation of several key actin-binding proteins, such as α-actinin, vinculin, gelsolin, and tropomyosins (TMs), is considered to contribute to the disorganized cytoskeleton present in many neoplastic cells. TMs stabilize actin filaments against the gel severing actions of proteins such as cofilin. Among multiple TMs expressed in non-muscle cells, tropomyosin-1 (TM1) isoform induces stress fibers and functions as a suppressor of malignant transformation. However, the molecular mechanisms of TM1-mediated cytoskeletal effects and tumor suppression remain poorly understood. We have hypothesized that the ability of TM1 to stabilize microfilaments is crucial for tumor suppression. In this study, by employing a variant TM1, which contains an N-terminal hemagglutinin epitope tag, we demonstrate that the N terminus is a key determinant of tropomyosin-1 function. Unlike the wild type TM1, the modified protein fails to restore stress fibers and inhibit anchorage-independent growth in transformed cells. Furthermore, the N-terminal modification of TM1 disorganizes the cytoskeleton and delays cytokinesis in normal cells, abolishes binding to F-actin, and disrupts the dimeric associations *in vivo*. The functionally defective TM1 allows the association of cofilin to stress fibers and disorganizes the microfilaments, whereas wild type TM1 appears to restrict the binding of cofilin to stress fibers. TM1-induced cytoskeletal reorganization appears to be mediated through preventing cofilin interaction with microfilaments. Our studies provide *in vivo* functional evidence that the N terminus is a critical determinant of TM1 functions, which in turn determines the organization of stress fibers.

One of the most common and yet prominent features of neoplastic cells is the presence of disorganized actin microfilaments (1, 2). A functionally defective cytoskeleton, arising from the disorganized microfilament architecture, has been shown to be responsible for the loss of normal cellular morphology and cell polarity; altered intracellular transport, cell motility, and cell adhesion; and defective cytokinesis. Suppression of several key actin-binding proteins, including tropomyosins (TMs),[1] occurs in many neoplastic cells, and this contributes to the assembly of disorganized cytoskeleton (reviewed in Refs. 3 and 4). Down-regulation of TMs in malignantly transformed cells has been known for about 2 decades and is widely reported (5–7), and yet the role of TMs in neoplastic transformation of cells remains incompletely understood.

TMs are a family of actin-binding proteins that stabilize microfilaments from the gel severing actions of proteins such as gelsolin and cofilin (1, 8). Multiple TMs are generated by alternative splicing with a high degree of tissue specificity. For example, fibroblasts express five different, closely related TMs that may be categorized into high and low M_r species containing 284 and 248 amino acids, respectively. TMs are key regulatory proteins of actin cytoskeleton in that they regulate almost all aspects of actin polymerization (9–13). Although the function of TMs is better elucidated in skeletal and cardiac muscles, given their diverse and tissue-specific expression patterns, the importance of the existence of multiple TMs in non-muscle cell physiology is poorly understood (8). It has been suggested that TM isoforms perform distinct functions rather than being simply redundant (1, 8). Work from this and several other laboratories shows that the high M_r TMs are consistently down-regulated in many malignantly transformed cells (5–7, 14–16). This suggests a role for TMs in the maintenance of normal growth and cytoskeletal organization, and that expression of TM1 may be incompatible with neoplastic growth.

In support of this hypothesis, we have recently shown that TM isoform-1 (TM1) expression is widely and profoundly down-regulated in primary breast tumors (17). Furthermore, TM1 restores the microfilament organization in transformed cells, and suppresses malignant growth (16, 18–20). Tumor suppression by TM1 is isoform-specific; for example, unlike TM1, closely related TMs such as TM2 failed to suppress transformed growth of highly malignant v-*ki-ras*-transformed NIH3T3 (DT) cells or MCF-7 human breast carcinoma cells (17, 19). Although these studies suggested TM1 is a class II tumor suppressor (17, 20, 21), the molecular basis of cytoskeletal organization and tumor suppression by TM1 remains unknown.

TM1 lacks distinct catalytic activity or binding partners that readily explain the isoform-specific anti-oncogenic effects. Structurally, all TMs are predominantly α-helical proteins in which hydrophilic and hydrophobic amino acids occupy defined positions in a repeating heptapeptide (22). TM1, like other TMs, is a cytosolic structural protein that binds to actin stoi-

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[1] The abbreviations used are: TMs, tropomyosins; DT, doubly transformed; HA, hemagglutinin; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; PMSF, phenylmethylsulfonyl fluoride; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

chiometrically in micromolar range. For example, both TM1 and TM2 bind to actin at 1:7 ratio, although some differences in binding properties exist (23). Nevertheless, it is intriguing that the biological effects of TM1 expression in tumor cells are remarkably different from those of other TM isoforms. We have considered that reorganization of microfilaments is a critical component of tumor suppression by TM1. The studies presented here demonstrate that the N terminus of TM1 is essential for TM1 functions and imply that TM1 is a key modulator of stress fibers.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—Culture conditions and media for NIH3T3, NIH3T3/TM1, and DT/TM1 cells have been described previously 2(4). Doubly transformed (DT) cells are NIH3T3 cells transformed with two copies of the v-*ki-ras* oncogene (7). An epitope-tagged TM1 was constructed by cloning TM1 in-frame in pCGN vector to add hemagglutinin (HA) epitope at the N terminus. The variant TM1 thus produced would have an N-terminal extension, ASSYPYDVPDYASLGGPSR, but contains identical wild type TM1 sequence from the "R" onward. DT cells were cotransfected with the recombinant plasmid and pCMV*neo* to generate single cell-derived clones by standard transfection methods. *N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate salts (Roche Applied Science) or LipofectAMINE (Invitrogen) were used for transfections. In experiments involving transient transfections, cells were routinely processed 48 h after transfection, unless otherwise indicated.

A TM polyclonal antibody, generated in this laboratory, that detects multiple TMs was described previously (16). TM311 mouse monoclonal antibody that recognizes a common epitope found in all high M_r TMs (25) was obtained from Sigma. HA (12CA5) mouse monoclonal antibody (Roche Applied Science) and antibodies against α-tubulin (Sigma), phosphocofilin (Upstate Biotechnology, Inc.), actin, and cofilin (Cytoskeleton, Inc., Denver, CO) were purchased.

Monolayer Growth and Morphology—Ten thousand cells were plated and counted, and cell numbers were plotted against time of culture. Morphology of subconfluent monolayer cultures was recorded by fixing and staining with a HEMA 3 kit obtained from Fisher. The samples were photographed at ×40 magnification.

Soft Agar Assays—Anchorage-independent experiments were performed in soft agar as described previously. One thousand cells were mixed in 0.36% agar, plated on a 0.8% agar base, and cultured for 10–14 days (18). Cells were stained overnight with 0.05% nitro blue tetrazolium in PBS, and colonies were counted.

Immunofluorescence—Cells were cultured in chamber slides (Nunc), fixed with 3.7% paraformaldehyde, and extracted with 0.5% Triton X-100 for 5 min (26). The samples were incubated with an appropriate primary antibody, followed by a second antibody conjugated to a fluorochrome, and finally with Texas Red-conjugated phalloidin. Slides were finally rinsed in water and mounted using Antifade kit (Molecular Probes). Images were recorded using a Zeiss LSM 510 confocal microscope and imported into Adobe Photoshop.

Immunoblotting and Immunoprecipitations—Cells were lysed in a buffer containing Nonidet P-40, sodium deoxycholate, and protease inhibitors and clarified at 14,000 × *g* (26). Supernatants containing 50–100 μg of proteins were separated on 13% SDS-polyacrylamide gels and immunoblotted. For immunoprecipitations, 200 μg of protein lysate, precleared with protein G for 1 h, was incubated with the primary antibody (27). The immune complexes were washed with immunoprecipitation buffer and subjected to SDS-PAGE. To quantify the protein expression, the exposed membranes were scanned, and the band intensities were calculated using the magic wand tool of the Adobe Photoshop (version 6.0).

Metabolic Labeling of Cells—Subconfluent cultures were pre-incubated with labeling medium (Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum) for 3 h. Metabolic labeling was carried out in the labeling medium containing [^{35}S]methionine (50 μCi/ml) for 6 h (24). Cells were washed with PBS and extracted in Nonidet P-40/deoxycholate lysis buffer. For immunoprecipitations and cross-linking experiments, lysates equivalent to 4×10^6 cpm were used.

F-actin Quantitation in Cells—F-actin content in cells was measured using a protocol described by Zigmond and co-workers (28) with minor modifications. Cells (1.5×10^5) were plated 12–24 h prior to experimentation. Monolayer cultures were washed with PBS, fixed, and stained in PBS containing 3.7% paraformaldehyde, 0.5% Triton X-100, and 0.2 μM TRITC phalloidin for 3 h at room temperature with constant rocking in the dark. Unincorporated TRITC phalloidin was removed by four PBS washes. Cell monolayer was extracted by using 1 ml of methanol, and the cell suspension was transferred to microcentrifuge tubes and incubated for 48 h with constant rocking at 4 °C. Cell debris was removed by centrifugation and fluorescence ($540_{ex}/575_{em}$) was read in an Aminco Bowman luminescence fluorimeter using the methanol solvent as blank. To determine background fluorescence, 2 μM of unlabeled phalloidin was added along with TRITC-phalloidin. To determine total actin content, cell lysates were probed with anti-actin antibody in immunoblots and expressed as a ratio with endogenous tubulin.

Cell Cycle Analysis—To estimate the fraction of cells in each phase of cell cycle, asynchronously growing subconfluent cultures were trypsinized and stained with 50 μg/ml propidium iodide in PBS containing 0.06% Nonidet P-40 and 30 μg/ml RNase A. Cell cycle analyses were carried out using a BD FACS Star Plus flow cytometer. Flow cytometry of serum-starved (24 h) or stimulated cells was performed. For serum stimulation, serum-starved cells were cultured in regular medium for 28 h.

HA-TM1 Purification—HA-TM1 was subcloned in pET3a (Novagen) and expressed in BL21 (DE3) pLysS bacteria. Cultures were grown to A_{600} 0.4–0.6 and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cells were harvested and resuspended for sonication in lysis buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1× protease inhibitor mixture (Roche Applied Science), 1 mg/liter DNase, 1 mg/liter RNase A, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 mM EDTA, 5 mM EGTA, 5 mM PMSF, 5 mM benzamidine, and 1 mg of potato carboxypeptidase inhibitor (Calbiochem) and immediately subjected to ammonium sulfate precipitation to achieve ~50% saturation. The protein pellet was resuspended and extensively dialyzed against a buffer containing 20 mM Tris (pH 7.5), 1 mM PMSF, and 1 mM benzamidine. The salt concentration of the dialysate was adjusted to 50 mM and subjected to DEAE-cellulose chromatography. HA-TM1 was eluted with 0.3 M NaCl containing buffer (20 mM Tris (pH 7.5), 5 mM EDTA and 2.5 mM PMSF), reprecipitated with ammonium sulfate (100% saturation to concentrate the eluate), and dialyzed. The protein sample was further purified by affinity chromatography on HA epitope affinity column (Roche Applied Science) as per the manufacturer's instructions. HA-TM1 was eluted with 1 mg/ml HA epitope peptide (Roche Applied Science) and dialyzed against the binding assay buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EGTA).

In Vitro Actin Binding Studies—Actin binding assays were performed as described previously (29) with modifications (30). HA-TM1 and wild type TM2 were cosedimented at 20 °C with chicken pectoral muscle F-actin (5 μM) in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and 0.5 mM dithiothreitol. The amounts of bound and free tropomyosin in the supernatants and pellets were quantitated by densitometry of SDS-polyacrylamide gels stained in Coomassie Blue using a Molecular Dynamics model 300A computing densitometer (Amersham Biosciences). To separate HA-TM1 from actin, the gels also contained 6 M urea. The free tropomyosin in the supernatants was calculated from standard curves for wild type tropomyosin. The curve for TM2 was fit using the Hill equation using SigmaPlot (SPSS Science, Chicago) that reported a K_{app}.

Cross-linking Studies—Homodimers of TM1 were stabilized by cross-linking with 5,5′-dithiobis-2-nitrobenzoic acid (24, 31) (DTNB) (Sigma), a sulfhydryl cross-linker, as described previously (24). Cell lysates were incubated with DTNB and were either subjected to immunoblotting (for unlabeled samples) or immunoprecipitation followed by SDS-PAGE and fluorography (for ^{35}S-labeled samples). Dimers are detectable when 2-mercaptoethanol is omitted in the gel sample buffer.

Statistical Analyses—Data are presented as mean ± S.D. from at least three independent determinations. *p* values were calculated by Student's two-tailed *t* test (32) using the software provided in Microsoft Excel (2002 edition).

RESULTS

In vitro studies have indicated that the N- and C-terminal ends of TMs are critical for binding to actin. Several studies have shown that some TMs, including TM1, require the acetylated N terminus for optimal binding to actin. N-terminal extensions, depending on the length and sequence, can alter TM functions (33–35). To elucidate the mechanism of TM1-mediated tumor suppression, we have modified the N-terminal end of TM1 molecule by introducing a hemagglutinin (HA) epitope. This epitope tag contains three prolines and would not be predicted to be α-helical. This tagged protein, referred to as

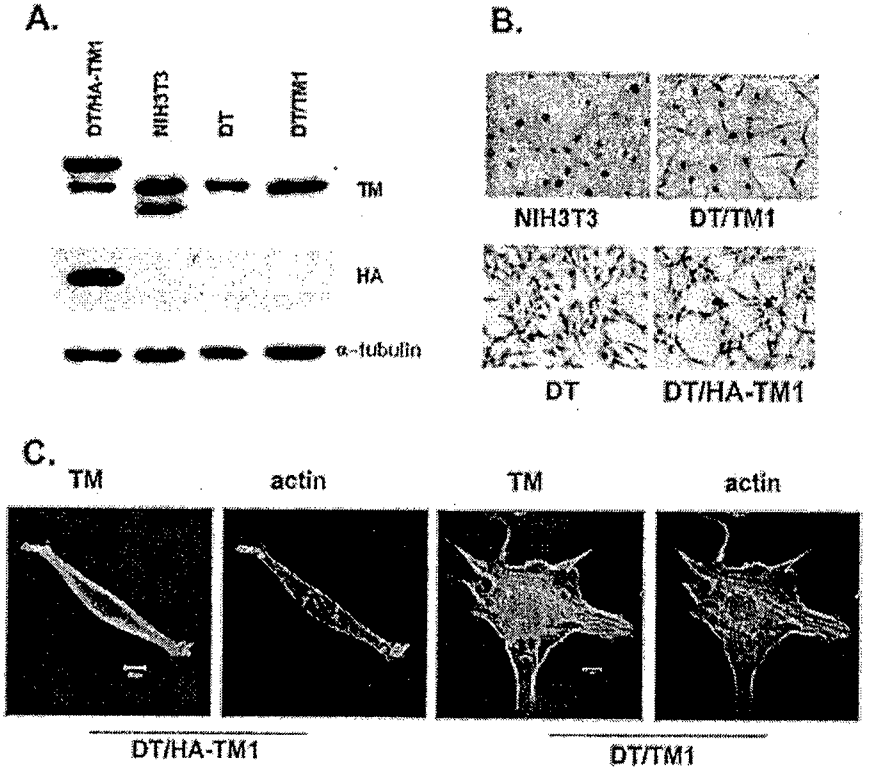

FIG. 1. **Effect of expression of HA-TM1 on DT cell morphology and microfilament organization.** *A*, TM profiles in the cell lines used in this study. Cell lysates were immunoblotted with either TM antibody or with HA antibody. *B*, morphology of cells grown in monolayer, as observed by light microscopy (×40 magnification). *C*, immunofluorescence microscopy. DT cells expressing either HA-TM1 or TM1 were stained with a TM polyclonal antibody that recognizes all TM isoforms and Texas Red-conjugated phalloidin to visualize F-actin. *Bar*, 10 μm.

HA-TM1, contains a 19-residue N-terminal extension, which otherwise is identical to the wild type protein, and retains all the actin binding domains of TM1. We tested the ability of HA-TM1 to regulate cytoskeletal organization and growth phenotype.

N-terminal Modification Abolishes TM1-mediated Cytoskeletal Reorganization and Tumor Suppression—We have used DT (NIH3T3 cells transformed by *v-Ki-ras*) cells as a model and transfected them with HA-TM1. Stable single cell clones, designated as DT/HA-TM1, were isolated. DT cells express TM1 at 50% levels compared with normal NIH3T3 fibroblasts, and TM2 and TM3 at essentially undetectable levels (18). Expression of HA-TM1 was detected by Northern blot (not shown) and immunoblot methods (Fig. 1*A*). The variant protein, because of the N-terminal extension, migrates slower than wild type TM1 on SDS-PAGE. The expression of endogenous TM1 in parental DT and DT/HA-TM1 cells is determined by the ratio of TM1: α-tubulin. In DT cells, the relative expression of TM1 was 0.7 ± 0.12, and in DT/HA-TM1 cells, the endogenous TM1 was expressed at 0.75 ± 0.08, indicating that the transfected HA-TM1 did not alter the levels of the endogenous wild type protein ($p < 1$).

Morphologically DT cells are spindle-shaped, lack stress fibers, and are not contact-inhibited. Because TM1 induces reorganization of the cytoskeleton to restore stress fibers with attendant cell spreading, we examined whether HA-TM1 alters the cell morphology. Morphologically, DT/HA-TM1 cells resembled parental DT and empty vector transfected cells. DT/HA-TM1 cells displayed spindle-shaped morphology, were not subject to contact inhibition and formed multiple foci (Fig. 1*B*). Confocal microscopy revealed that HA-TM1 expression, unlike that of the wild type TM1 protein (18), did not induce the formation of stress fibers (Fig. 1*C*).

Because TM1 induces cytoskeletal reorganization with the assembly of stress fibers, it is likely that enhanced TM1 levels may increase the levels of F-actin. Therefore, we examined whether TM1 up-regulates F-actin levels and whether the inability of the variant protein to reorganize cytoskeleton is reflected in lower F-actin content. Overnight cultures of NIH3T3 cells, DT cells, DT/TM1, and DT/HA-TM1 cells were fixed, and the F-actin content was determined using TRITC-conjugated phalloidin. The fluorescent intensities were normalized to 1.5×10^5 cells (Table I). Consistent with the degree of microfilament organization, NIH3T3 cells contained most F-actin. DT and DT/HA-TM1 cells contained comparable amounts of F-actin, but exhibited significantly lower F-actin than NIH3T3 cells, as measured by fluorescence intensity ($p < 0.02$). Enhanced expression of TM1 resulted in the reemergence of microfilaments, which reflected in increased F-actin content. F-actin content was significantly higher in DT/TM1 cells (1.97 ± 0.32) compared with DT cells (1.01 ± 0.24) ($p < 0.03$). However, the total actin content in DT-derived cells was unchanged, when quantitated by immunoblotting and expressed relative to tubulin (Table I).

TABLE I
TM1 enhances the cellular F-actin content

F-actin content was determined by measuring the fluorescence of TRITC-labeled phalloidin, as described under "Experimental Procedures." Means ± S.D. are derived from three independent experiments. Relative fluorescence was normalized to 1.5×10^5 cells. Total actin and tubulin content were measured in 50 μg of protein from the indicated cell lines by immunoblotting. The ratio of actin to tubulin is taken as a measure of total actin content. The *p* values reported in the text are calculated by two-tailed paired *t* test.

Cell type	F-actin content	Total actin content
	fluorescence units	*actin/tubulin*
NIH3T3	3.63 ± 0.81	1.36 ± 0.11
DT	1.01 ± 0.24	1.15 ± 0.2
DT/TM1	1.97 ± 0.32	1.18 ± 0.11
DT/HA-TM1	0.72 ± 0.01	1.13 ± 0.12

Next we determined whether HA-TM1 altered the growth properties of DT cells. Variant TM1 did not affect either the monolayer growth or anchorage-independent growth rates of DT cells (Fig. 2, *A* and *B*). In contrast, wild type TM1 signifi-

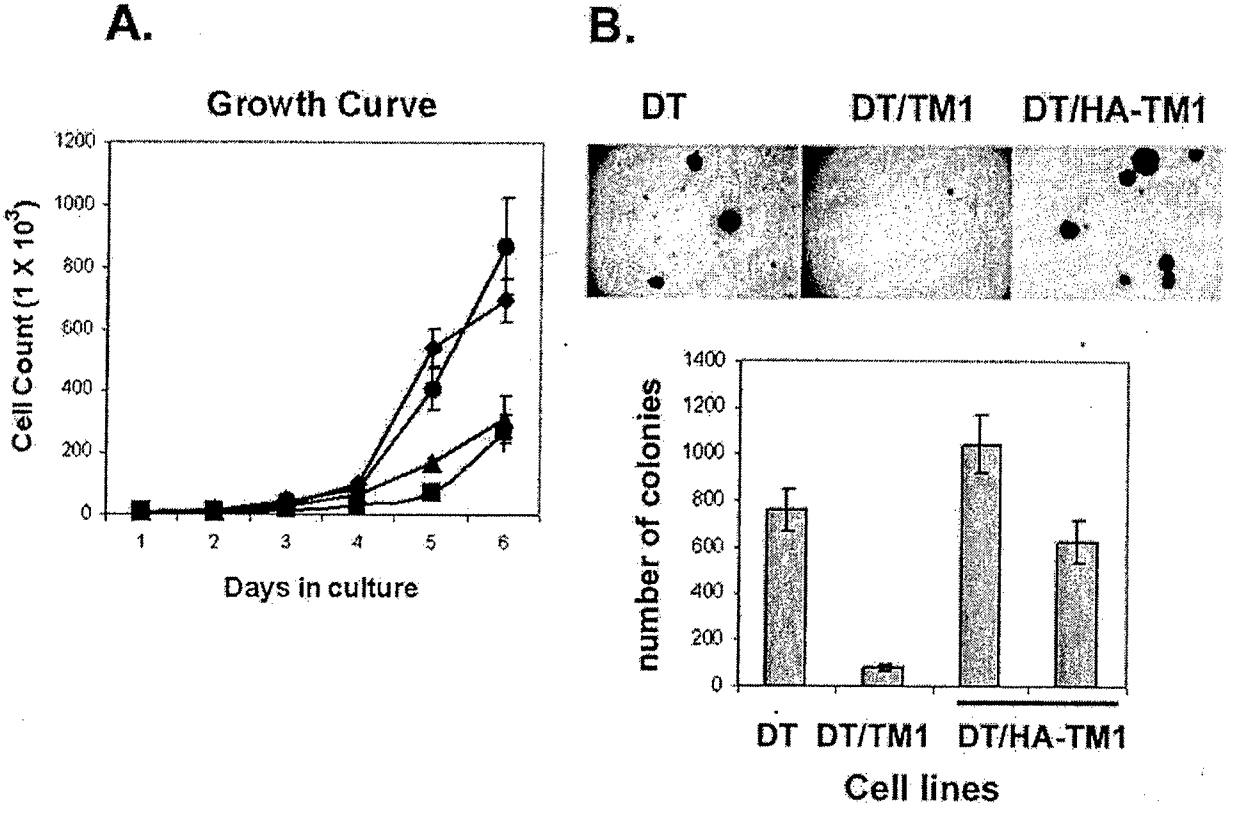

FIG. 2. **N-terminal modification abolishes anti-oncogenic effects of TM1.** Growth of cells in monolayer (*A*) or under anchorage-independent conditions (*B*) was measured. *A*, the following cell lines were used: DT/TM1 (■), NIH3T3 (▲), DT/HA-TM1 (♦), and DT (●). The *p* values were calculated using two-tailed *t* test assuming unequal variance. Photomicrographs of anchorage-independent cultures (*B*) are shown. The *histogram* shows the efficiency of colony formation in DT, two independent clones of DT/HA-TM1 and DT/TM1 cells. The *error bars* indicate mean ± S.D. from triplicate samples.

cantly decreased monolayer growth compared with HA-TM1 ($p < 0.005$), comparable with the NIH3T3 cells. Consistent with its inability to alter the cytoskeletal organization and morphology, HA-TM1 expression did not affect the anchorage-independent growth of DT cells. DT/HA-TM1 cells grew rapidly and formed colonies in soft agar as efficiently as the parental DT cells or vector control cells. In contrast, DT/TM1 cells failed to grow under anchorage-independent growth conditions (18). Thus, the N-terminal modification of TM1 abolishes the ability of TM1 to induce cytoskeletal reorganization and inhibits its anti-oncogenic properties.

HA-TM1 Disrupts Microfilaments in Normal Fibroblasts—Next, we investigated whether the modification of the N terminus of TM1 would interfere with the ability of TM1 to associate with existing normal microfilament structures. NIH3T3 cells were transiently transfected with HA-TM1 (Fig. 3*A*). HA-TM1 incorporated into microfilaments and colocalized with F-actin. The HA-TM1 was distributed uniformly throughout the cytoskeleton, a finding consistent with a previous report (27). However, examination of the microfilament architecture after extended periods (48–72 h) revealed severe disruptions in microfilaments (Fig. 3*B*). Cells expressing HA-TM1 generally lacked well defined linear microfilaments that traverse the cell, which are typical of NIH3T3 cells. Instead, the transfected cells displayed wavy and disorganized microfilaments containing the variant protein and F-actin filaments, and aberrant cellular morphology when compared with the untransfected cells. These findings suggest that HA-TM1 associates with microfilaments and subsequently perturbs the cytoarchitecture (Fig. 3*B*).

Transient transfection typically produces an overabundance of the gene product, which could potentially result in artifacts. In this case, enhanced expression of a TM protein could perturb intracellular TM pools, leading to aberrant cytoskeleton, an effect independent of the N-terminal modification of TM1. To rule out the effects of overabundance of the transfected protein, we have examined the microfilament organization in NIH3T3 cell lines that were stably transduced with TM1 (NIH3T3/TM1 cells) (24). Enhanced expression of TM1 did not disorganize microfilaments, as did the variant protein (Fig. 3*C*). TM1 colocalized with linear, well defined stress fibers, and the normal cellular morphology was not compromised in NIH3T3/TM1 cells. These results indicate that enhanced TM1 expression *per se* does not induce disorganized cytoskeleton. Because TMs dimerize in "head to tail" fashion and associate with actin, modification of the N terminus of TM1 could have interfered with either dimerization or binding to actin, or both.

To investigate further the effects of the N-terminal modification of TM1 molecule, we have stably transfected NIH3T3 cells with HA-TM1 (Fig. 3*D*). Immunofluorescence experiments show that microfilaments are often "pushed" to a side, and a substantial amount of variant TM1 occupies the perinuclear area. Variant TM1 is also colocalized with phalloidin-positive microfilaments, indicating that during dynamic reorganization of stress fibers, the HA-TM1 associates with filaments. The variant protein, because of its abundance, may compete with the endogenous TM1, although the F-actin binding and dimerization properties of TM1 and variant TM1 significantly differ (see below). However, it is possible that HA-TM1 and endogenous TM1 coexist in the stress fibers.

Light microscopic observation of NIH3T3/HA-TM1 cells revealed the presence of high number of binucleated cells, indi-

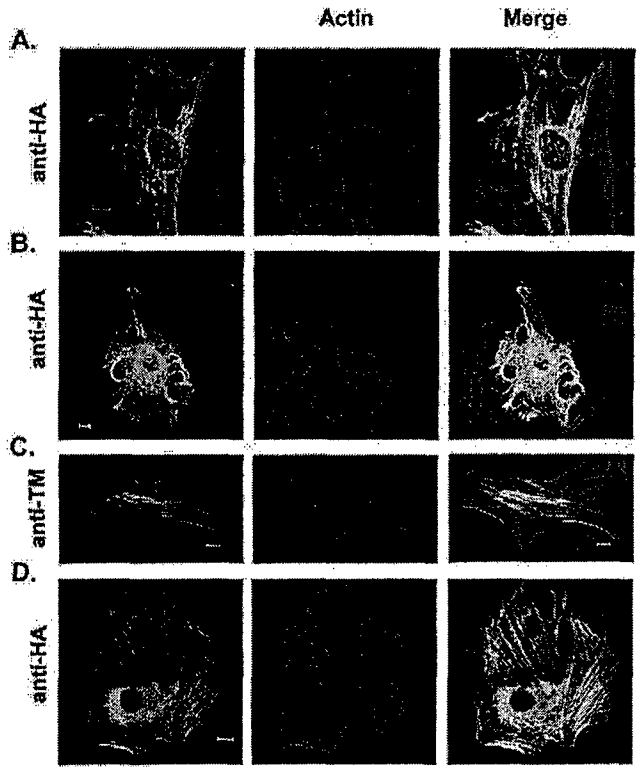

FIG. 3. **Microfilament organization in HA-TM1-expressing cells.** Immunofluorescence was performed to detect the expression of HA-TM1 (anti-HA antibody) or TMs (TM antibody) and F-actin (phalloidin). NIH3T3 cells transiently transfected with HA-TM1 were stained at 24 (*A*) or 48–72 h (*B*). Stable cell lines of NIH3T3/TM1 cells (*C*) and NIH3T3/HA-TM1 (*D*) cells are also depicted. *Bar*, 10 μm.

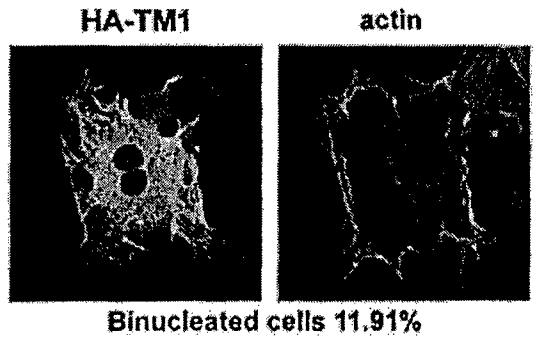

FIG. 4. **HA-TM1 increases hypertetraploid cells.** A representative binucleated, stable NIH3T3/HA-TM1 cell stained with anti-HA antibody and phalloidin is depicted. The numbers of binucleated cells were counted and the percentage is given. In control NIH3T3 cells the number of binucleated cells is ≤1%.

cating defects in cytokinesis in the transfected cells (Fig. 4). Asynchronously growing populations of NIH3T3/HA-TM1 cells contained as high as 11.91% (43 of 361 cells) of binucleated cells compared with ≤1% (5 of 550 cells) found with the unmodified cells. Flow cytometry revealed a significantly higher number of cells with >4 N DNA content (Table II). NIH3T3, NIH3T3/TM1, and NIH3T3/HA-TM1 cells were analyzed for cell cycle progression under normal, serum-starved, and serum-stimulated conditions. Although the NIH3T3 and NIH3T3/TM1 cells did not differ in the number of hypertetraploid cells (2.9 ± 0.2 and 3.16 ± 0.03, respectively), NIH3T3/HA-TM1 cells contained 10.3 ± 1.38% cells with >4 N DNA content. Thus, the number of hypertetraploid cells in NIH3T3/HA-TM1 was significantly higher compared with parental and wild type TM1-expressing cells ($p < 0.01$). The number of hypertetraploid cells obtained by flow analysis is comparable with the number of binucleated cells obtained by visual counting (above). Serum starvation resulted in the accumulation of cells in G_0-G_1 phase in all three cell types. Although NIH3T3 and NIH3T3/TM1 cells contained a smaller percentage of cells in G_2-M phase in starved samples (7.96 ± 0.31% and 10.12 ± 0.36%, respectively), the percentage of cells in G_2-M phase in NIH3T3/HA-TM1 was much higher (26.52 ± 1.4%; $p < 0.001$ compared with NIH3T3 and NIH3T3/TM1 cells). Significantly, the number of cells with >4 N DNA content in NIH3T3/HA-TM1 cells dramatically decreased when the progression of cell cycle was inhibited by deprivation of serum (1.96 ± 0.46%). Serum stimulation, however, led to an increase in the hypertetraploid populations in NIH3T3/HA-TM1 cells (7.4 ± 0.35%) but not in NIH3T3 or NIH3T3/TM1 (0.98 ± 0.37 and 2.24 ± 0.22%, respectively; $p < 0.001$ compared with NIH3T3/HA-TM1 cells). Collectively, these results suggest that the cytokinesis was slower in NIH3T3/HATM1 cells and that perturbing the intracellular TM1 pool interferes with normal cytokinesis.

The NIH3T3 cell lines selected for stable expression of the HA-TM1 extinguish the expression of the transfected TM1 upon a few passages, indicating that sustained expression of HA-TM1 is not compatible with cell growth. Whereas expression of HA-TM1 induces cytoskeletal disorganization and delays cytokinesis, it does not promote anchorage-independent growth in NIH3T3 cells (data not shown).

Variant TM1 Does Not Bind to F-actin—TM1 is a major actin-binding protein, and the functions of TM1, including suppression of transformed growth, may be dependent upon its ability to bind to and stabilize actin microfilaments. Therefore, we have examined HA-TM1 binding to actin *in vitro*. HA-TM1 was expressed in bacteria and purified, and actin-binding properties of the isolated protein were determined by cosedimentation. As a positive control for actin binding, we used unacetylated, recombinant (*Escherichia coli*-expressed) rat TM2. Unacetylated rat TM2 bound well to actin, with a binding constant in the micromolar range (23, 30, 33), similar to that of acetylated TM1. Unacetylated TM1 binds F-actin with lower affinity (23). Fig. 5 shows that TM2 bound to F-actin in a concentration-dependent and saturable manner with a K_{app} of 2×10^6 M^{-1}, consistent with published work (30). In contrast, HA-TM1 bound poorly to F-actin, too weakly to obtain a binding constant. Although the effect of other N-terminal extensions on TM1 function has not been investigated, it is well established that introduction of various N-terminal extensions on striated muscle α-tropomyosin overcomes the requirement for N-terminal acetylation for actin binding (29, 34, 35). In addition, striated muscle α-tropomyosin with N-terminal fusions, including HA, can incorporate into actin-containing structures in living cells (36, 37). Whereas the mechanism by which the HA epitope adversely influences TM1-actin affinity is unclear, the results are consistent with the inability of HA-TM1 to induce microfilaments and to suppress transformed growth, as well as with its interference with cytokinesis.

Dimerization and Interactions between Wild Type and Variant TM1 Proteins—TMs bind to actin as parallel, in register dimers (38, 39). Previous studies from this laboratory indicate that homodimers of TM1 form a stable component in cytoskeletal compartment in DT/TM1 cells (24). We examined whether actin binding of the variant TM1 is influenced by the ability of TM1 to associate as dimers, and whether dimerization is important in TM1-induced cytoskeletal reorganization.

First, we tested whether HA-TM1 forms homodimers by cross-linking the unique cysteine residue with a sulfhydryl cross-linker, DTNB, to stabilize the dimers in the lysates of metabolically labeled DT/HA-TM1 cells (Fig. 6*A*). Cross-linked

TABLE II
N-terminal modification of TM1 induces hypertetraploid

The percent fraction of cells in each phase of the cell cycle was analyzed from either actively growing subconfluent cells or from serum-starved cultures for 24 h. Starved cultures were stimulated for 28 h. Accumulation of hypertetraploid cells is significantly higher in HA-TM1 cells compared with those expressing wild type TM1 or unmodified NIH3T3 cells. Data are shown as means ± S.D. from triplicate samples of a representative experiment. The p values reported in the text are calculated by two-tailed t test, assuming unequal variance.

Cell type-growth condition	% fraction of cells			
	G_0-G_1	S	G_2-M	Hypertetraploid cells
NIH3T3-active	36.75 ± 2.48	29.25 ± 5.18	33.98 ± 2.97	2.9 ± 0.2
NIH3T3-serum-starved	89.5 ± 0.53	2.53 ± 0.28	7.96 ± 0.31	0.46 ± 0.05
NIH3T3-stimulated	65.81 ± 6.03	25.25 ± 4.37	8.93 ± 1.93	0.98 ± 0.37
NIH3T3/TM1-active	28.44 ± 1.27	40.12 ± 1.17	31.43 ± 0.35	3.16 ± 0.03
NIH3T3/TM1-serum-starved	85.1 ± 0.61	4.77 ± 0.6	10.12 ± 0.36	0.64 ± 0.1
NIH3T3/TM1-stimulated	56.05 ± 0.27	25.88 ± 0.54	18.06 ± 0.34	2.24 ± 0.22
NIH3T3/HA-TM1-active	36.86 ± 3.57	31.47 ± 4.42	31.62 ± 1.83	10.3 ± 1.38
NIH3T3/HA-TM1- serum-starved	71.51 ± 2.06	1.96 ± 0.78	26.52 ± 1.4	1.96 ± 0.46
NIH3T3/HA-TM1-stimulated	42.38 ± 3.84	19.12 ± 2.97	38.46 ± 1.28	7.4 ± 0.35

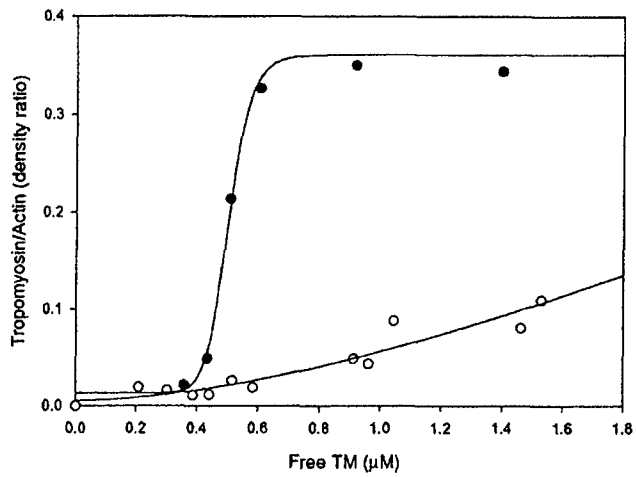

FIG. 5. **N-terminal modification decreases TM1 binding to F-actin.** Actin binding of bacterially purified HA-TM1 (○) is measured by cosedimentation, as described under "Experimental Procedures." Wild type TM2 (●) is used as a control, which bound to actin with an affinity of K_{app} 2×10^6 M^{-1}. The TM/actin density ratio (an arbitrary number) *versus* free TM is plotted. The value at saturation for wild type TM2 represents stoichiometric saturation (1 TM:7 actins).

samples were immunoprecipitated with either TM antiserum or monoclonal anti-HA antibody and analyzed by SDS-PAGE, either in the presence or absence of 2-mercaptoethanol in gel sample buffer by SDS-PAGE. In DT and DT/TM1 cells, homodimers of TM1 were readily evident when samples were analyzed using a buffer lacking 2-mercaptoethanol. The HA-TM1 protein expressed in DT/HA-TM1 cells, however, remained as monomer. However, by cross-linking using unlabeled cells, which measures steady state interactions, dimers of HA-TM1 were detected by both TM and HA antibodies in immunoblotting (Fig. 6*B*). These results indicate that nascently synthesized HA-TM1 either compartmentalizes differently or folds improperly preventing dimeric associations. Furthermore, HA-TM1 isolated from bacteria (same preparation used for binding assays, see above) also cross-linked into dimers (data not shown), indicating that dimerization alone may not ensure proper actin binding.

Next we investigated whether the wild type and variant TM1 proteins interact with each other. Coimmunoprecipitation experiments were performed using metabolically labeled (Fig. 6*C*) and unlabeled cell lysates (Fig. 6*D*) to detect interactions at steady state. As expected, both proteins were immunoprecipitated with TM antibody. In labeled DT/HA-TM1 cells, however, the tagged protein did not coimmunoprecipitate with the endogenous protein as judged by immunoprecipitation with anti-HA antibody, suggesting a TM1:HA-TM1 heterodimer is not formed (Fig. 6*C*). Immunoprecipitation of unlabeled DT/HA-TM1 and NIH3T3/HA-TM1 cell proteins with anti-HA antibody resulted in coimmunoprecipitation of a modest amount of endogenous TM1, indicating a small amount of heterodimer formation (Fig. 6*D*).

Interestingly, during our efforts to isolate HA-TM1 from DT/HA-TM1 cells, we found that as the purification progressed, the enrichment of HA-TM1 on HA affinity columns also yielded the endogenous TM1, resulting in isolation of HA-TM1 and TM1 complexes (data not shown). This finding suggests that although HA-TM1 is capable of binding to TM1, such interactions do not occur efficiently in cells (Fig. 6, *C* and *D*).

TM1, but Not Variant TM1, Alters Cofilin Distribution—Stress fiber organization is controlled by Rho kinase, which regulates phosphorylation of myosin light chain kinase and LIM kinase. Whereas increased phosphorylation of myosin light chain kinase promotes contractility leading to microfilament reorganization, activated LIM kinase phosphorylates cofilin at Ser-3 and inhibits its severing action (reviewed in Ref. 40). Previous studies from this laboratory have shown that the Rho kinase pathway is essential for TM1-induced microfilament reorganization in *ras*-transformed cells (26). Down-regulation of LIM kinase, which appears to result in a significant increase in activated cofilin, has been implicated as a key mechanism in cytoskeletal disruption in *ras*-, and *src*-transformed cells (41, 42). Tropomyosins protect microfilaments from the severing and depolymerizing actions of cofilin (43, 44). Therefore, we considered whether TM1-induced cytoskeletal reorganization involves inhibition of cofilin-mediated microfilament depolymerization.

We measured activation status of cofilin in NIH3T3, DT, DT/TM1, and DT/HA-TM1 cells using phosphocofilin-specific antibodies. Normal NIH3T3 cells contained consistently the highest phosphocofilin to cofilin ratio, which indicates the least activity (Fig. 7*A*). The ratio of phosphocofilin:cofilin in NIH3T3 cells is taken as 100%. The ratio of phosphocofilin to total cofilin is significantly lower in DT (57.3 ± 11.6%, $p < 0.05$) and DT/HA-TM1 (68.9 ± 13.7%, $p < 0.03$) cells compared with NIH3T3 cells (100%) in five different experiments. However, TM1 expression in DT cells modestly elevated phosphocofilin content. In DT/TM1 cells phosphocofilin to cofilin ratio was found to be at 79.43 ± 15.49% when compared with NIH3T3 cells ($p < 0.02$) but does not appear to be significantly different from DT/HA-TM1 cells ($p < 0.1$). These results suggest that down-regulation of cofilin activity alone may not completely account for TM1-induced cytoskeletal organization.

Next, we have examined whether the subcellular distribution of cofilin is altered in TM1-induced cytoskeletal reorgani-

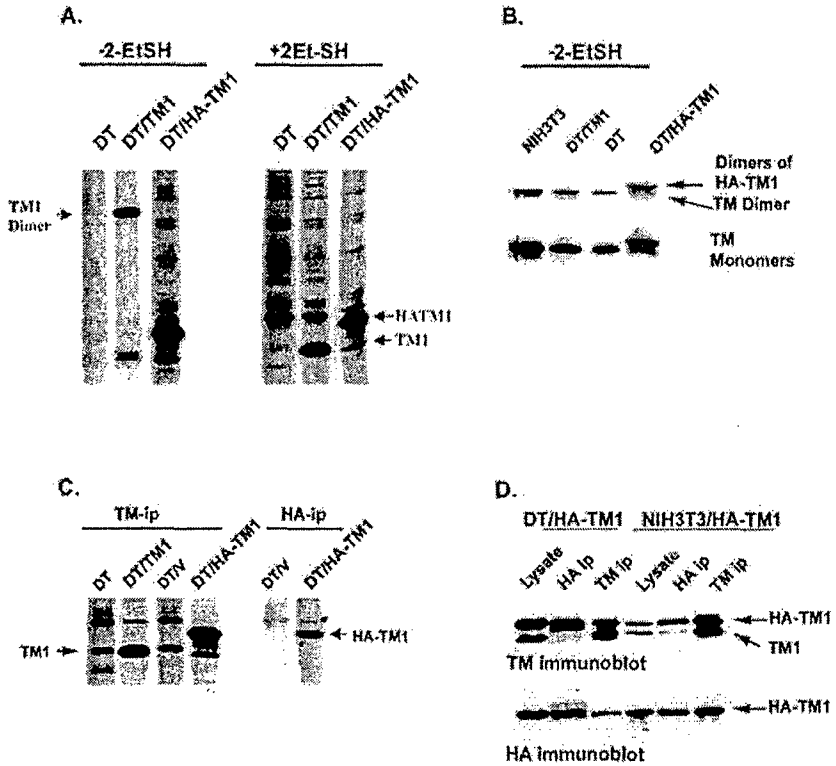

FIG. 6. **Dimerization and interactions of HA-TM1.** *A*, metabolically labeled cell lysates were cross-linked with DTNB, immunoprecipitated, and analyzed without or with 2-mercaptoethanol (*EtSH*) in gel sample buffer. TM1 dimers, monomeric TM1, and HA-TM1 are identified. *B*, unlabeled cell lysates (steady state) were cross-linked and immunoblotted with TM antibody. Dimeric and monomeric TMs are marked. *C*, immunoprecipitation (*-ip*) of TM1 and HA-TM1 in metabolically labeled cells. Although TM antibody immunoprecipitates TM1 and HA-TM1, the epitope tag antibody does not coimmunoprecipitate wild type TM1, indicating a lack of heterodimer formation. *D*, HA-TM1 interacts weakly with TM1 at steady state. Cell lysates from NIH3T3/HA-TM1 and DT/HA-TM1 were immunoprecipitated with TM or HA antibodies and immunoblotted, as indicated. Cell lysates were also run as controls.

zation. In NIH3T3 cells, cofilin is distributed throughout the cytoplasm, as well as in the nucleus (Fig. 7*B*). Cofilin does not appear to associate with either F-actin or TMs present in stress fibers. In DT/HA-TM1 cells, however, cofilin and TMs are distributed in the cytoplasm; similar staining was found in DT cells (data not shown). In contrast, in DT/TM1 cells, which contain well developed microfilaments, TMs and cofilin do not colocalize, and the staining pattern resembles that of NIH3T3 cells.

Because high M_r TMs, including TM1, bind and stabilize microfilaments against the action of cofilin, we investigated whether HA-TM1-induced microfilament disruption is mediated by cofilin. NIH3T3 cells were transfected with HA-TM1 to test whether cofilin and variant TM1 colocalize. At earlier time points (24–48 h) of transfection, we could detect cofilin association with HA-TM1-positive aberrant microfilaments (Fig. 7*C*, *top panel*). However, the residual normal microfilaments lacked detectable staining of cofilin and HA-TM1. As the transfection time progressed, both of these proteins were localized to the perinuclear area, which lacked defined microfilament structures. In such cells stress fibers were displaced to the periphery and were stained positive for phalloidin alone. Cofilin and HA-TM1 were not detectable in those stress fibers (Fig. 7*C*, *middle panel*). It is likely that the linear filaments protected by the endogenous TM1 may exclude cofilin binding to the stress fibers. Transfection of DT cells with HA-TM1, however, did not result in the formation of microfilaments, and cofilin colocalized with the HA-TM1 throughout the cytoplasm (Fig. 7*C*, *bottom panel*). These results suggest that modified TM1, which interacts poorly with F-actin, allows cofilin binding to microfilaments and remodels the cytoskeleton.

DISCUSSION

The important findings of this investigation are as follows. 1) The integrity of the N terminus of TM1 is critical for TM1-induced microfilament reorganization and tumor suppression. 2) Modification of the N terminus decreases TM1 binding to actin which allows cofilin-mediated cytoskeletal disorganization. 3) TM1 is required for normal cytokinesis. Suppression of TM1 in normal cells is difficult due to its abundance, and to date no mutants of TM1 have been described. Hence, the variant TM1 is a useful tool to study TMs, as it functions as a dominant negative mutant.

In this study we have focused on structural aspects of TM1 that mediate cytoskeletal reorganization and tumor suppression. It is intriguing that although TMs bind to F-actin in the micromolar range, and TM1 has no unique structural domains, TM1 exerts remarkably specific effects on cell growth compared with other TMs (17, 19, 45, 46). Subtle differences in TM-actin interactions modulate a range of cellular events that depend on cytoskeletal organization (1). Many *in vitro* binding studies, however, have shown that the N and C termini are critical determinants of TM binding to actin (47, 48). Some TMs, for instance, striated muscle α-tropomyosin and yeast TM, require N-terminal acetylation, which may stabilize the N terminus (49) for actin binding (23, 35, 50) and for the function of yeast TM *in vivo* (51, 52). The requirement for striated muscle α-tropomyosin and yeast TM for *N*-acetylation may be overcome by extending the N terminus by addition of a variety of peptide sequences (29, 34, 35, 37, 50). However, this is not the case with HA-TM1.

Other efforts to obtain insight into cellular TM functions have also examined the contribution of the N and C termini of TMs, utilizing chimeric TMs. Chimeras of non-muscle and muscle type TMs (TM5/3), consisting of TM5 and TM3, have been shown to bind F-actin avidly and cause accumulation of multinucleated cells resulting from delayed cytokinesis (48). Most interesting, cells expressing the chimera retained normal microfilament architecture. More recent studies using transgenic expression of chimeras of α and β TMs have identified that the C terminal portion of TM is an important determinant of cardiac function (53).

Because TMs bind cooperatively in a head to tail fashion, modification of N terminus is sufficient to disrupt TM1 association with actin, even though the rest of the coding sequence

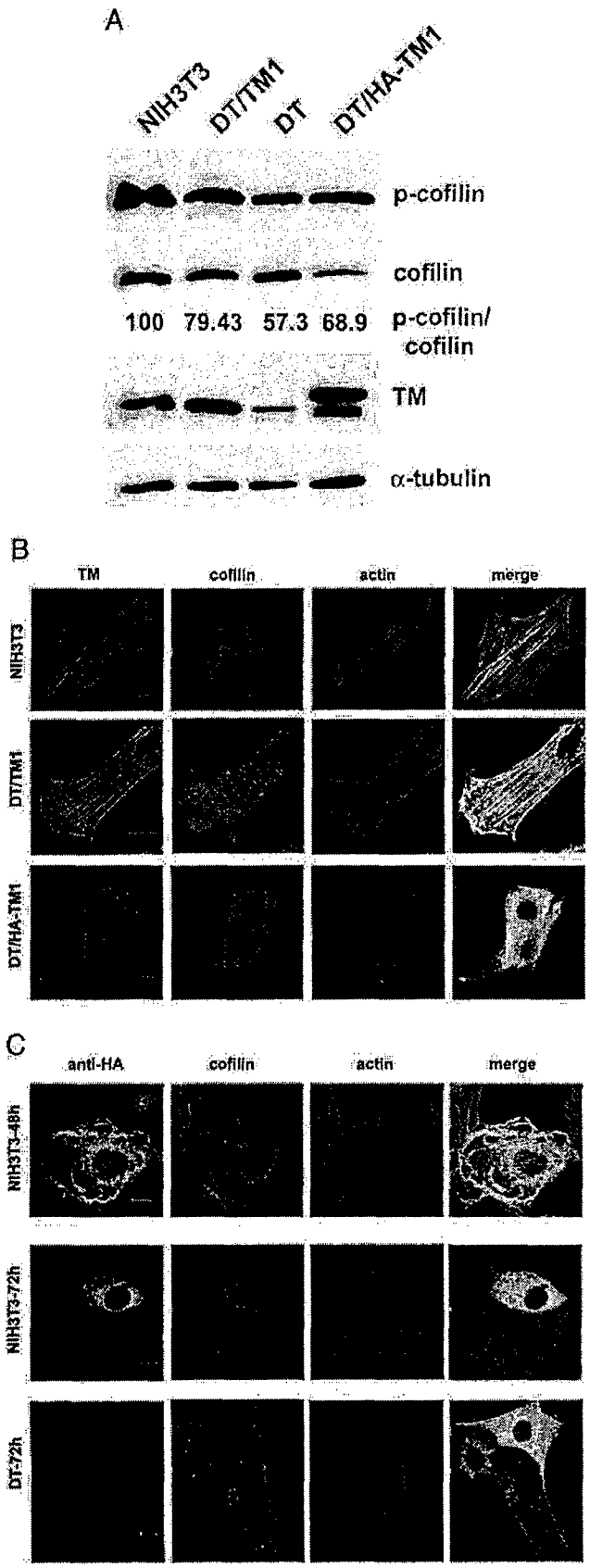

FIG. 7. **TM1, but not modified TM1, alters cofilin distribution.** *A*, cellular phosphocofilin content is down-regulated in DT and DT/HA-TM1 cells. Cell lysates were immunoblotted with an antibody that recognizes phosphocofilin or total cofilin. The blot was also probed with a TM polyclonal antibody and anti-α-tubulin antibody. The ratio of phosphocofilin to total cofilin was calculated. The results (mean values) of five independent experiments, normalized to NIH3T3 as 100%, are presented as the phosphocofilin/cofilin ratio. The *p* values are calculated, based on paired two-tailed *t* test. *B*, TM1 and F-actin do not colocalize with cofilin. Indicated cell lines were stained for TM (using TM311 antibody), cofilin and F-actin. *C*, HA-TM1 and cofilin colocalize. NIH3T3 and DT cells were transiently transfected with HA-TM1 and stained to detect HA-TM1, cofilin, and F-actin. At earlier points of transfection (*top panel*), HA-TM1 and cofilin colocalize in transfected cells and appear to be excluded from microfilaments. Upon continued expression of the variant protein, stress fibers are displaced to the periphery of the cell, whereas cofilin and HA-TM1 colocalize to the cell body (*middle panel*). DT cells were also transfected with HA-TM1 and observed at 72 h (*bottom panel*). Cofilin and HA-TM1 were distributed throughout the cell body in transfected and untransfected DT cells. *Bar*, 10 μm.

and actin binding domains are identical to the wild type protein. The abundantly produced variant TM1, notwithstanding its lower affinity to actin, competes with the endogenous protein, associates with microfilaments, and subsequently disrupts microfilament architecture. Alternatively, HA-TM1 may compete for caldesmon (54), and thus could impact TM1-actin interactions, which also could disrupt the stability of the cytoskeleton.

Interaction of Wild Type and HA-TM1—Homodimers of TM1 are incorporated into the cytoskeletal compartment. The dimeric TM1 is hypothesized to be important for cytoskeletal reorganization and tumor suppression (24). It is interesting that although HA-TM1 retains the ability to dimerize and associate with endogenous TM1 *in vitro*, it fails to bind to F-actin in the cosedimentation experiments. Coimmunoprecipitation and cross-linking studies (Fig. 6) show that HA-TM1 homodimerizes and can interact *in vitro* with the endogenous TM1, a finding consistent with earlier studies (27). *In vivo*, however, HA-TM1 does not associate with the endogenous TM1, nor does the nascently synthesized protein dimerize. Several possibilities may explain this discrepancy.

First, N-terminal modification may alter subcellular localization of TM1. HA-TM1 segregates into a distinct subcellular location from microfilaments in NIH3T3 cells (Figs. 3*D* and 7*C*). Second, altered conformation and/or the inability to be recognized for certain post-translational modifications could account for the failure of the variant TM1 to be sorted with the endogenous TM1.

TM1 Regulates Cytokinesis—Because microfilaments provide the necessary force for cytokinesis (55), and the stability of actin microfilaments is controlled by TMs, alterations in TM-actin interactions could impact cell division. Expression of HA-TM1 remarkably increases the number of binucleated cells, indicating aberrant cytokinesis (Fig. 4 and Table II). Similar to the results reported here, a previous study showed that chimeric TMs exhibit higher binding affinity to F-actin and induce defective cytokinesis. These data suggest that the exact matching of the N and C termini is essential for normal functioning of TMs (48). Further work showed that the hTM5/3 chimeras induce altered motile behavior during the cytokinesis (56). These workers suggested the stronger binding of the chimeras with actin (57) would differentially regulate the contractile ring than would the wild type TMs. The defective cytokinesis could be the consequence of a generation of much higher force for separating daughter cells through interaction between the chimeras and actin than that produced by the wild type proteins (56). Because HA-TM1 interacts poorly with actin, HA-TM1 binding could yield lower than necessary force and hence delay the cytokinesis. This is supported by the fact that blockade of the cell cycle through serum starvation results in the completion of cytokinesis in HA-TM1 cells with a decrease in hypertetraploid populations. Serum starvation of NIH3T3/HA-TM1

cells, however, produces a higher percentage of cells in G_2-M phase, compared with the parental and wild type TM1-expressing cells, indicating a slower cytokinesis.

Other researchers also report that high M_r TMs localize to the contractile ring during cell division (58). Collectively, these results suggest that both high and low M_r TMs are required for the normal completion of cytokinesis.

Role of Cofilin in Variant TM1 Induced Cytoskeletal Changes—TMs protect actin filaments from the gel severing and depolymerization actions of ADF/cofilin and gelsolin (12, 43, 59). Investigations into TM1-induced microfilament organization suggested that total gelsolin levels are not altered in TM1-expressing DT cells, suggesting that gelsolin may not be involved (26). Cofilin and TMs bind to actin in a mutually exclusive manner, and cofilin increases rate of depolymerization from the pointed end of actin filament and severs actin filaments (reviewed in Ref. 13). Recent studies have suggested that some TM isoforms may bind to cofilin (60) and inhibit actin nucleation and branching (11). Furthermore, genetic studies in *Caenorhabditis elegans* show that CeTM inhibits ADF/cofilin-mediated thin filament dynamics (43). LIM kinases, one of the effectors of Rho kinase signaling pathways, phosphorylate and inactivate cofilin (61–63). Our previous work (26) shows that Rho kinase signaling pathways are required to maintain the TM1-induced cytoskeleton, which indicates a role for cofilin in TM1-induced cytoskeletal dynamics.

We find that *ras* transformation decreases the phosphocofilin content (Fig. 7*A*), indicating that enhanced activation of cofilin may reshape the deregulation of cytoskeletal organization into a more dynamic and motile cytoskeleton. These results are in agreement with the published reports that show that neoplastic transformation uncouples Rho signaling pathways, enhancing cofilin activity (41, 42). Restoration of TM1 expression modestly elevates the phosphocofilin content in DT cells, indicating that additional mechanisms such as the altered intracellular compartmentalization may limit cofilin activity in TM1-induced cytoskeleton in *ras*-transformed cells. Support for this possibility comes from the immunofluorescence experiments that show that cofilin does not colocalize with F-actin in DT/TM1 cells or NIH3T3 cells, which contain well defined stress fibers. Functionally defective TMs, such as HA-TM1, however, appear to be unable to protect microfilaments, as evidenced by colocalization of cofilin in aberrant microfilaments. The variant TM1 and cofilin colocalize and are absent in normal stress fibers. Thus, TM1 is a key protein in stabilizing stress fibers against the action of cofilin-mediated remodeling. Similarly, TMs are suggested to limit cofilin to the leading edge of the cells, and where localized, dynamic remodeling of actin filaments occurs (44).

Alternate mechanisms of cytoskeletal reorganization by TM1 in *ras*-transformed cells, involving post-translational modifications, cannot be ruled out. Extracellular signal-regulated kinase-mediated phosphorylation of TM1 is suggested to be a signal for the assembly of stress fibers in endothelial cells (64). Because extracellular signal-regulated kinase signaling is constitutively activated in DT cells and the variant TM1 expression is unable to rescue stress fiber assembly, further work is needed to clarify the role of intracellular signaling in TM1-mediated cytoskeletal reorganization in transformed cells. Although we have shown that TM1 reorganizes microfilaments in oncogene-transformed cells (18, 20) and breast carcinoma cells (16, 17), other investigators have found that TM1 is unable to reorganize microfilaments in *ras*-transformed RIE cells (65) and neuroblastoma cells (66), suggesting a requirement for additional and as yet unknown factors in TM1-induced cytoskeletal reorganization.

In summary, we have identified that N-terminal integrity is a key regulator of TM1 functions. TM1-mediated cytoskeletal reorganization and tumor suppression may be dependent on TM1-F-actin interactions as well as on restricting the access of gel-severing proteins to actin filaments. Furthermore, TM1-induced cytoskeletal reorganization may involve inhibiting the gel severing activity of cofilin through modulation of phosphorylation status and preventing its association with microfilaments. Our results also suggest that TM1 is pivotal in maintaining the stress fibers.

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REFERENCES

1. Lin, J. J., Warren, K. S., Wamboldt, D. D., Wang, T., and Lin, J. L. (1997) *Int. Rev. Cytol.* **170,** 1–38
2. Pawlak, G., and Helfman, D. M. (2001) *Curr. Opin. Genet. Dev.* **11,** 41–47
3. Button, E., Shapland, C., and Lawson, D. (1995) *Cell Motil. Cytoskeleton* **30,** 247–251
4. Janmey, P. A., and Chaponnier, C. (1995) *Curr. Opin. Cell Biol.* **7,** 111–117
5. Hendricks, M., and Weintraub, H. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 5633–5637
6. Matsumura, F., Lin, J. J., Yamashiro-Matsumura, S., Thomas, G. P., and Topp, W. C. (1983) *J. Biol. Chem.* **258,** 13954–13964
7. Cooper, H. L., Feuerstein, N., Noda, M., and Bassin, R. H. (1985) *Mol. Cell. Biol.* **5,** 972–983
8. Pittenger, M. F., Kazzaz, J. A., and Helfman, D. M. (1994) *Curr. Opin. Cell Biol.* **6,** 96–104
9. Wen, K. K., Kuang, B., and Rubenstein, P. A. (2000) *J. Biol. Chem.* **275,** 40594–40600
10. Strand, J., Nili, M., Homsher, E., and Tobacman, L. S. (2001) *J. Biol. Chem.* **276,** 34832–34839
11. Blanchoin, L., Pollard, T. D., and Hitchcock-DeGregori, S. E. (2001) *Curr. Biol.* **11,** 1300–1304
12. Ishikawa, R., Yamashiro, S., and Matsumura, F. (1989) *J. Biol. Chem.* **264,** 16764–16770
13. Cooper, J. A. (2002) *Curr. Biol.* **12,** R523–R525
14. Bhattacharya, B., Prasad, G. L., Valverius, E. M., Salomon, D. S., and Cooper, H. L. (1990) *Cancer Res.* **50,** 2105–2112
15. Novy, R. E., Lin, J. L., Lin, C. S., and Lin, J. J. (1993) *Cell Motil. Cytoskeleton* **25,** 267–281
16. Mahadev, K., Raval, G., Bharadwaj, S., Willingham, M. C., Lange, E. M., Vonderhaar, B. K. V., Salomon, D., and Prasad, G. L. (2002) *Exp. Cell Res.* **279,** 40–51
17. Raval, G. N., Bharadwaj, S., Levine, E. A., Willingham, M. C., Geary, R. L., Kute, T., and Prasad, G. L. (2003) *Oncogene* **22,** 6194–6203
18. Prasad, G. L., Fuldner, R. A., and Cooper, H. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7039–7043
19. Braverman, R. H., Cooper, H. L., Lee, H. S., and Prasad, G. L. (1996) *Oncogene* **13,** 537–545
20. Prasad, G. L., Masuelli, L., Raj, M. H., and Harindranath, N. (1999) *Oncogene* **18,** 2027–2031
21. Sers, C., Emmenegger, U., Husmann, K., Bucher, K., Andres, A. C., and Schafer, R. (1997) *J. Cell Biol.* **136,** 935–944
22. Perry, S. V. (2001) *J. Muscle Res. Cell Motil.* **22,** 5–49
23. Pittenger, M. F., Kistler, A., and Helfman, D. M. (1995) *J. Cell Sci.* **108,** 3253–3265
24. Prasad, G. L., Fuldner, R. A., Braverman, R., McDuffie, E., and Cooper, H. L. (1994) *Eur. J. Biochem.* **224,** 1–10
25. Nicholson-Flynn, K., Hitchcock-DeGregori, S. E., and Levitt, P. (1996) *J. Neurosci.* **16,** 6853–6863
26. Shah, V., Bharadwaj, S., Kaibuchi, K., and Prasad, G. L. (2001) *Oncogene* **20,** 2112–2121
27. Gimona, M., Watakabe, A., and Helfman, D. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9776–9780
28. Tardif, M., Huang, S., Redmond, T., Safer, D., Pring, M., and Zigmond, S. H. (1995) *J. Biol. Chem.* **270,** 28075–28083
29. Heald, R. W., and Hitchcock-DeGregori, S. E. (1988) *J. Biol. Chem.* **263,** 5254–5259
30. Hammell, R. L., and Hitchcock-DeGregori, S. E. (1996) *J. Biol. Chem.* **271,** 4236–4242
31. Lehrer, S. S., and Joseph, D. (1987) *Arch. Biochem. Biophys.* **256,** 1–9
32. Glantz, S. A. (1997) *Primer of Biostatistics*, 4th Ed., McGraw-Hill Inc., New York
33. Cho, Y. J., Liu, J., and Hitchcock-DeGregori, S. E. (1990) *J. Biol. Chem.* **265,** 538–545
34. Monteiro, P. B., Lataro, R. C., Ferro, J. A., and Reinach, F. (1994) *J. Biol. Chem.* **269,** 10461–10466
35. Urbancikova, M., and Hitchcock-DeGregori, S. E. (1994) *J. Biol. Chem.* **269,** 24310–24315
36. Ranucci, D., Yamakita, Y., Matsumura, F., and Hitchcock-DeGregori, S. E. (1993) *Cell Motil. Cytoskeleton* **24,** 119–128
37. Michele, D. E., Albayya, F. P., and Metzger, J. M. (1999) *J. Cell Biol.* **145,** 1483–1495
38. McLachlan, A. D., and Stewart, M. (1975) *J. Mol. Biol.* **98,** 293–304
39. Lehrer, S. S. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 3377–3381
40. Dos Remedios, C. G., Chhabra, D., Kekic, M., Dedova, I. V., Tsubakihara, M., Berry, D. A., and Nosworthy, N. J. (2003) *Physiol. Rev.* **83,** 433–473

41. Pawlak, G., and Helfman, D. M. (2002) *J. Biol. Chem.* **277,** 26927–26933
42. Pawlak, G., and Helfman, D. M. (2002) *Mol. Biol. Cell* **13,** 336–347
43. Ono, S., and Ono, K. (2002) *J. Cell Biol.* **156,** 1065–1076
44. DesMarais, V., Ichetovkin, I., Condeelis, J., and Hitchcock-DeGregori, S. E. (2002) *J. Cell Sci.* **115,** 4649–4660
45. Takenaga, K., and Masuda, A. (1994) *Cancer Lett.* **87,** 47–53
46. Gimona, M., Kazzaz, J. A., and Helfman, D. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9618–9623
47. Moraczewska, J., Nicholson-Flynn, K., and Hitchcock-DeGregori, S. E. (1999) *Biochemistry* **38,** 15885–15892
48. Warren, K. S., Lin, J. L., McDermott, J. P., and Lin, J. J. (1995) *J. Cell Biol.* **129,** 697–708
49. Greenfield, N. J., and Hitchcock-DeGregori, S. E. (1995) *Biochemistry* **34,** 16797–16805
50. Maytum, R., Geeves, M. A., and Konrad, M. (2000) *Biochemistry* **39,** 11913–11920
51. Polevoda, B., Cardillo, T. S., Doyle, T. C., Bedi, G. S., and Sherman, F. (2003) *J. Biol. Chem.* **278,** 30686–30697
52. Singer, J. M., and Shaw, J. M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 7644–7649
53. Jagatheesan, G., Rajan, S., Petrashevskaya, N., Schwartz, A., Boivin, G., Vahebi, S., Solaro, R. J., Labitzke, E., Hilliard, G., and Wieczorek, D. F. (2003) *J. Biol. Chem.* **278,** 23204–23211
54. Sobue, K., and Sellers, J. R. (1991) *J. Biol. Chem.* **266,** 12115–12118
55. Glotzer, M. (2001) *Annu. Rev. Cell Dev. Biol.* **17,** 351–386
56. Wong, K., Wessels, D., Krob, S. L., Matveia, A. R., Lin, J. L., Soll, D. R., and Lin, J. J. (2000) *Cell Motil. Cytoskeleton* **45,** 121–132
57. Novy, R. E., Sellers, J. R., Liu, L. F., and Lin, J. J. (1993) *Cell Motil. Cytoskeleton* **26,** 248–261
58. Hughes, J. A., Cooke-Yarborough, C. M., Chadwick, N. C., Schevzov, G., Arbuckle, S. M., Gunning, P., and Weinberger, R. P. (2003) *Glia* **42,** 25–35
59. Ishikawa, R., Yamashiro, S., and Matsumura, F. (1989) *J. Biol. Chem.* **264,** 7490–7497
60. Bryce, N. S., Schevzov, G., Ferguson, V., Percival, J. M., Lin, J. J.-C., Matsumura, F., Bamburg, J. R., Jeffrey, P. L., Hardeman, E. C., Gunning, P., and Weinberger, R. P. (2003) *Mol. Biol. Cell* **14,** 1002–1016
61. Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caroni, P. (1998) *Nature* **393,** 805–809
62. Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999) *Science* **285,** 895–898
63. Zebda, N., Bernard, O., Bailly, M., Welti, S., Lawrence, D. S., and Condeelis, J. S. (2000) *J. Cell Biol.* **151,** 1119–1128
64. Houle, F., Rousseau, S., Morrice, N., Luc, M., Mongrain, S., Turner, C. E., Tanaka, S., Moreau, P., and Huot, J. (2003) *Mol. Biol. Cell* **14,** 1418–1432
65. Shields, J. M., Mehta, H., Pruitt, K., and Der, C. J. (2002) *Mol. Cell. Biol.* **22,** 2304–2317
66. Yager, M. L., Hughes, J. A., Lovicu, F. J., Gunning, P. W., Weinberger, R. P., and O'Neill, G. M. (2003) *Br. J. Cancer* **89,** 860–863

APPENDIX 6

Manuscript in revision

Re-sensitization of breast cancer cells to anoikis by Tropomyosin-1 through cytoskeleton-dependent modulation of integrin activity and Rho kinase signaling.

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Summary:

Two of the most common properties of malignant cells are the presence of aberrant actin cytoskeleton and resistance to anoikis. Suppression of several key cytoskeletal proteins, including tropomyosin-1 (TM1), during neoplastic transformation is hypothesized to contribute to the altered cytoskeleton and neoplastic phenotype. Using TM1 as a paradigm, we have shown that cytoskeletal proteins induce anoikis in breast cancer (MCF-7 and MDA MB 231) cells. Here, we have tested the hypothesis that TM1-mediated cytoskeletal changes regulate integrin activity and the sensitivity to anoikis. TM1 expression in MDA MB MB231 cells promotes the assembly of stress fibers, induces rapid anoikis via caspase dependent pathways involving cytochrome C. Further, TM1 inhibits binding of MDA MB 231 cells to collagen I, but promotes adhesion to laminin. Inhibition of Rho kinase disrupts TM1-mediated cytoskeletal reorganization and adhesion to the extracellular matrix components, whereas the parental cells attach to collagen I, spread and form extensive actin meshwork in presence of Rho kinase inhibitor, underscoring the differences in malignant and TM1-transduced breast cancer cells. Further, treatment with the cytoskeletal disrupting drugs rescues the cells from TM1-induced anoikis. These new findings demonstrate that that aberrant cytoskeleton contributes to neoplastic transformation by conferring resistance to anoikis. Restoration of stress fiber network through enhanced expression of key cytoskeletal proteins may modulate the activity of focal adhesions and sensitize the neoplastic cells to anoikis.

Introduction:

It is now well established that the interactions of cells with their surroundings exert profound influence on cell phenotype (1-5). The growth and differentiation of normal epithelial cells is dependent on survival signals that are activated by the interactions of adhesion molecules and the extra cellular matrix compounds (ECM) (6,7). Under normal growth conditions, integrins are activated following binding to ECM, which in turn, activates a number of diverse intracellular events (8). These events include the assembly of focal adhesions, actin microfilament reorganization and recruitment of a number of signaling molecules to the focal adhesions. Although the mechanisms need to be completely delineated, it is suggested that the reorganized cytoskeleton is believed to transduce integrin-derived signals to promote growth and differentiation (9). However, cells rapidly undergo apoptosis when deprived of the adhesion-derived signals, in a process termed anoikis (6). Consequently, cell survival is ensured only when cells are in their physiological environment. Thus, anoikis plays a key role during normal growth and differentiation, as illustrated in tubulogenesis of mammary epithelial cells (10,11) and wound healing (12).

Most malignant cells, however, are resistant to anoikis, and thus are able to survive in a non-physiological environment to grow as metastases. Some studies have suggested that resistance to anoikis is acquired early on during the neoplastic growth (10,13). Several mechanisms have been suggested to contribute to the resistance of anoikis in malignant cells. For example, activation of oncogenes (14), growth factors (15,16) and modulation of integrin activity (17-19), which result in the activation of a plethora of mitogenic and survival signaling, have been shown to confer resistance to

anoikis in malignant cells. However, few studies have examined the mechanisms/agents that re-sensitize tumor cells to anoikis. For instance, inactivation of tumor suppressor genes, such as PTEN (20,21) and $p16^{INK4a}$ (22,23), also are associated with the acquisition of resistance to anoikis.

One of the most prominent features of malignant cells is the presence of altered actin cytoskeleton, arising from the suppression of several key actin-binding proteins, such as profilin, α-actinin, tropomyosins (TMs) (Reviewed in (24,25)). The aberrant cytoskeleton contributes to neoplastic growth in several ways including through altered cell motility (reviewed in(26-28)) and sensitivity to apoptosis (29). However, it is unresolved whether the tumor cell cytoskeleton plays a role in conferring adhesion-independent survival.

Work from this and other laboratories has identified that the tropomyosin isoform-1 (TM1), is consistently suppressed in breast cancer cell lines (30) and tissues (31), and is downregulated in urinary bladder tumors (32), suggesting that the loss of TM1 may contribute to the neoplastic transformation. Restoration of TM1 expression in several oncogene-transformed (33-35) and spontaneously transformed breast cancer cells (31,36) reorganizes microfilaments, forms stress fibers in a Rho-kinase-dependent fashion (37) and suppresses the anchorage-independent growth. Further investigations have revealed that TM1 induces anoikis in breast cancer cells, and thus may suppress their malignant behavior (31,36). Since TMs stabilize actin cytoskeleton (reviewed in (38)) and regulate actin-myosin interactions (reviewed in (39)), we have hypothesized that TM1 mediated cytoskeletal reorganization is critical for the anti-neoplastic effects of TM1 (40).

We have sought to determine whether the cytoskeletal organization modulates the sensitivity to anoikis. We have utilized TM1-mediated cytoskeletal changes as a paradigm to investigate the mechanisms of resensitization of tumor cells to anoikis. Given the predominant role of integrins in adhesion-dependent signaling, and that TM1 promotes detachment-induced apoptosis, we have found that TM1 modulates the expression and activity of integrins in breast cancer cells, which may be important in TM1-induced anoikis.

Materials and Methods:

Cell lines and reagents: Culture conditions for MDA MB231 and MDA MB231/TM1 cells have been previously described (31). The extra cellular matrix (ECM) components, rat tail collagen-I, collagen-IV, mouse laminin I and fibronectin were purchased from BD Biosciences. Poly-L-Lysine was obtained from Sigma. Anti-integrin antibodies for determining integrin expression were obtained from Chemicon: α2 (P1E6), β1 (clone HB1.1), α6 (clone NKI-GoH3) and α9 (Y9A2). The Integrin antibodies direct to β4 and α6 subunits (439-9B and 135-13C) were from Rita Falcioni (41). Caspase-3, caspase-7, caspase-8 and PARP were purchased from Cell Signaling. Caspase inhibitor, Z-Val-Ala-Asp-CH_2F (Z-VAD), was purchased from Calbiochem and the stock solution prepared in DMSO. In experiments involving reagents dissolved in DMSO or ethanol, treatment with the vehicle alone was used as controls. Texas-red conjugated phalloidin was obtained from Molecular Probes.

Immunoblotting: Cytoplasmic cell lysates were prepared by NP40/DOC buffer (40) and total cell lysates were prepared using SDS buffer (2% SDS, 10mM Tris-HCl pH8.0), 1mM sodium vanadate, 10mM sodium fluoride and 100μM β-glycerophosphate). The SDS-solubilized lysates were sonicated with a Branson sonifier-250 (6 pulses with microtip at 50% duty cycle and output control at 5) to reduce viscosity. Gel electrophoresis was carried out under non-reduced conditions for detecting integrins α2, and β1. TM expression was determined by a pan-TM antibody prepared in house (36). We also used anti-peptide raised against a specific sequence of TM1 molecule that does not reacts with other TM molecules (36). The membranes were probed with antibodies against α–tubulin or β-actin (Sigma) as load controls.

Cell adhesion assays. Twenty-four well culture plates (Costar) were coated with 200µl of ECM components (collagen-I, collagen-IV, laminin or fibronectin) at a concentration of 50µg/ml for 1hr at room temperature with constant shaking. Poly-L-Lysine (100µg/ml) was used for determining non-integrin mediated adhesion. The coated wells were washed thrice with PBS and blocked in 200µl of 2%BSA in PBS for 1hr at 37°C. The blocked wells were washed thrice with PBS. Cell adhesion assay was performed by plating $2x10^5$ cells in 1.0ml of serum containing medium for 30min at 37°C as described by Dickson's group (42). Briefly, the attached cells were stained with 200µl of crystal violet solution (0.05% w/v in 25% methanol) for 10min, washed thrice by immersion in water and air-dried. The samples were solubilized in 500µl of 0.1M sodium citrate solution and 50% ethanol (v/v) for 5min and the absorbance was read at 540nm using a Bio-Rad Smartspec 3000 spectrophotometer. Photomicrographs of bound cells were captured on a Zeiss inverted microscope with a 40X objective.

In experiments involving the use of inhibitors or antibodies, cells were pretreated for 60 min prior to the adhesion assay. The Rho kinase inhibitor, Y-27632 (Yoshitomi Pharmaceuticals) was used at 20µM, (TS2/16 (Pierce) at 6µg/ml and Latranculin A (Sigma), dissolved in ethanol, at 0.5µM. Cells were treated with etoposide at 25 µM for 48h to determine the susceptibility of the cells to apoptosis. To determine the specificity of integrin binding to a particular ECM, inhibitory integrin antibodies (Chemicon) were used. The integrins and the appropriate blocking or stimulating antibodies, in parenthesis, are as follows: α2 (clone P1E6), β1 (clone P4C10), β1 stimulatory (TS2/16) for collagen-

I binding; α6 (clone NKI-GoH3) and β4 (clone ASC-3) for laminin at a concentration of 4μg/ml.

Immunofluorescence Experiments: Cells were plated on ECM coated chamber slides (Nunc) and allowed to adhere for 30min at 37°C. Cells were pretreated with 20μM of Y-27632 compound for 30min., as needed. After 30min of binding, the slides were gently rinsed twice with PBS to remove unattached cells and fixed using 3.7% paraformaldehyde for 10min. Cells were extracted with 0.5% TritonX-100 for 5min, followed by three washes with PBS before blocking and hybridization with antibodies as described previously (40). The samples were mounted using slow fade (Molecular Probes) to prevent fading. Images of the stained cells were captured using Zeiss LSM 510 confocal microscopy and imported into Adobe Photoshop (version 6.0).

Flow cytometry: To determine the cell surface expression of integrins sub-confluent cell cultures were trypsinized, neutralized with media and were washed with PBS. All incubations were performed on ice to prevent internalization of the antibody. Cells were blocked in PBS containing 1% BSA for 1h, incubated with integrin antibody (20μg/ml) for another hour. The cells were collected by centrifugation (1000xg), washed twice with PBS containing 0.1%BSA and incubated in the dark with fluorochrome conjugated secondary antibody for 1h. Finally, the unbound secondary antibody was removed by washing thrice with PBS containing 0.1 %BSA and resuspended at a concentration of $1x10^6$cells/ml. The stained cells were immediately analyzed by flow cytometry (BD FACS Star Plus).

Cell death assays: Cells ($5x10^5$) were plated on poly-(2-hydroxyethyl methacrylate) (Poly-HEMA) (Sigma) coated dishes (31) in serum containing media, unless otherwise indicated. At the end of the culture (usually 6h or 24h), cells were collected and several assays to determine apoptosis were performed. The DNA content of subG0-G1 phase of cell cycle was determined using propidium iodide staining of ethanol fixed cells (31). Annexin V staining (BioVision) was performed according to manufacturer's instruction and analyzed by flow cytometry. The activation of caspases was monitored by immunoblotting. Cell counts were also taken at intervals to confirm cell death. Nuclear morphology of cells was determined by cytospinning 0.5 - 1.0 x 10^4 cells on a slide and staining with DAPI (Sigma) at a concentration of 10ng/ml (31).

For visualizing cytochrome-c release, a published protocol (43) was used with some modifications. Briefly, cells grown in suspension for defined periods of time were cytospun onto slides and fixed with 3.7% paraformaldehyde for 10min. After 3 washes with PBS, the cells were permeabilized with 0.05% saponin in PBS for 5min followed by ice cold acetone for 10min. After three washes with PBS, the slide was blocked with 1%BSA in PBS for 30min, incubated for 90min with 1:50 dilution of anti-cytochrome-c (clone 6H2.B4) in block solution and followed by 1:50 dilution of Rhodamine conjugated goat-anti mouse (Molecular Probes) in block solution for 1h. The cells were then postfixed for 10min in 3.7% paraformaldehyde containing 0.1mg/ml of Hoechst 33342 stain (Molecular Probes), and processed as described for immunofluorescence experiments.

For determining the effect soluble ECM components (collagen-I, fibronectin, laminin and collagen-IV) in anoikis, poly-HEMA coated plates were used. $5x10^5$cells/ml

was incubated with or without ECM at a concentration of 20μg/ml for 24h. Cells were collected and the anoikis was measured by flow cytometry (31).

Results:

TM1 promotes microfilament re-organization and cell-spreading in breast cancer cells: TM1-induced anoikis is markedly rapid in MDA MB 231 cells in serum free media, and we have used this cell type as a model (31). Since TM1 is a microfilament-stabilizing protein that promotes stress fiber assembly (33,35,36), we have first examined whether TM1 reorganizes microfilaments in MDA MB 231 cells. Parental MDA MB 231 cells, which lack TM1 expression (30,36,44) are spindle shaped cells and grow as dispersed cells in monolayer cultures while, restoration of TM1 protein levels produces marked changes in the cellular morphology. The cells transduced with TM1 (MDA MB 231/TM1) (Figure 1A) appear well-spread and grow as groups of cells, features that are consistent with normal epithelial cell growth (Figure 1B). Whereas the parental cells lack stress fibers, TM1 expression reorganizes cytoskeleton, with the assembly of stress fibers (Figure 1C). Cells transfected with empty vector (MDA MB 231/V cells) resembled the parental cells morphologically (not depicted), and behaved similarly in the following experiments as the unmodified MDA MB 231 cells. We routinely analyze two independent clones of MDA MB 231/TM1 cells.

TM1 induces anoikis in breast cancer cells in normal growth media: Previously we have shown that transduction of breast cancer cells with TM1 induces anoikis when cultured in serum free-media (31). Since both growth factor- and adhesion-derived signaling pathways are necessary for normal cell survival, and malignant cells appear to primarily dependent on growth factors (serum) for survival (7), we examined whether TM1-induced anoikis can be overcome by culturing in normal growth media. The cells were cultured in serum free medium (31) or in presence of regular growth media containing

10% fetal bovine serum on poly HEMA-coated plates for 6 and 24 h, and the anoikis was determined by measuring the DNA content in the subG_0-G_1 phase of the cell cycle. The survival of unmodified MDA MB 231 cells in suspension cultures is not affected by the presence or the absence of serum, as indicated by the background levels of DNA in the subG_0-G_1 phase of cell cycle (Table 1). TM1 expression, however, induced significant anoikis by 6h in serum free as well as in normal growth conditions, and massive apoptosis was detected by 24h, and therefore all the subsequent experiments were performed in regular growth medium.

TM1-induced anoikis was further confirmed by fragmented nuclear morphology (Figure 2A and B), increased annexin V staining (Figure 2C) and PARP cleavage (Figure 2D). These data suggest that TM1 renders breast cancer cells dependent on adhesion-derived survival signals, and growth factors (serum) fail to abrogate TM1-induced anoikis. To test that the apoptotic machinery is not defective and remains functional, MDA MB 231/TM1 cells were treated with etoposide to induce cell death. As shown in Figure 2D (lanes marked ET), treatment of both parental and TM1-expressing cells resulted in apoptosis, as measured by the cleavage of PARP.

TM1-induced anoikis is mediated by the intrinsic death pathway: Anoikis is known to be mediated through the extrinsic (death receptor-mediated) and the intrinsic (mitochondrial) pathways of cell death (45,46). To investigate the mechanism of TM1-induced anoikis, we have determined the activities of the caspases that mediate cell death pathways. As shown in Figure 3A, cell detachment triggers the activation of caspases 3 and 7 in MDA MB 231/TM1 cells by 6h of suspension culture, whereas the adherent cells do not contain the active caspases. The unmodified MDA MB 231 cells lack caspase

activation in monolayer or in suspension, which is in agreement with their resistance to anoikis. Treatment of the parental and MDA MB 231/TM1 cells with etoposide however, activated the caspases leading to apoptosis of the adherent cells. Further, we find that the mitochondrial integrity of MDA MB 231/TM1 cells cultured in suspension is breached, as evident from the loss of staining and the release of cytochrome C into the cytoplasm (Figure 3B, bottom panels). Consistent with the role of caspases in anoikis, inhibition of caspases with a broad specificity inhibitor rescued MDA MB.231/TM1 cells from anoikis (Figures 3C and 3D). Collectively, these data suggest that TM1-induces anoikis through the intrinsic death pathways.

TM1 modulates the expression and the activities of integrins in breast cancer cells: Deprivation of integrin-derived survival signals are believed to trigger anoikis (reviewed in (6,47)). Consistently, modulation of the expression and the activities of integrins have been known to alter the susceptibility of cells to anoikis (17-19). To investigate whether TM1-induced anoikis is mediated through modulation of integrin expression/activity, we first determined the adhesion of MDA MB 231/TM1 cells to the ECM components.

MDA MB 231 cells and the vector control cells bound to collagen very efficiently (Figure 4A). The binding of MDA MB 231/TM1 cells to collagen, however, was significantly suppressed (50-70%). In contrast, the cell adhesion to laminin was markedly (about 3 fold) enhanced by TM1 expression, whereas binding to fibronectin was modestly enhanced. Non-integrin mediated binding, as measured by adhesion to poly L-lysine-coated surfaces was comparable in all the cell lines. Further, we examined whether TM1-mediated inhibition of adhesion to collagen I was specific by testing binding to collagen I and collagen IV. Adhesion of MDA MB 231/TM1 cells to collagen

I was inhibited, whereas the parental and TM1-transduced cells bound equally well to collagen IV (Figure 4B).

We next investigated whether the differential binding properties of MDA MB 231/TM1 cells results in distinct organization of microfilaments. As shown in Figures 4C and 4D, parental MDA MB 231 cells spread well on collagen I and laminin, form lamellipodia and stain intensely for cortical F-actin. MDA MB 231/TM1 cells, upon attachment to ECM, form stress fibers, indicating that TM1 primarily promotes the formation of stress fibers even under conditions of poor cell adhesion as observed on collagen I. These data indicate that TM1 expression modulates cell adhesion to the ECM components, possibly through regulating the expression and activity of integrins.

Cell adhesion to ECM is mediated through the heterodimeric transmembrane integrin receptors. Since TM1 modulates adhesion to collagen I and laminin, we investigated whether the expression of collagen- and laminin-binding integrins is altered. The $\alpha_2\beta_1$ integrin is a major collagen receptor (48) and is prominently expressed in MDA MB 231 cells (42). Flow cytometric analysis showed that both α_2 and β_1 subunits are present on MDA MB 231 cells. The cell surface expression of β_1 integrin in MDA MB 231/TM1 was comparable to that obtained with the parental cells, whereas the levels of α_2 integrin were markedly downregulated, indicating that the downregulation of α_2 integrin correlated with the reduced adhesion of these cells to collagen I (Figure 5A). In agreement with flow cytometry data, immunoblotting shows that the α_2 integrin was downregulated in TM1-transduced cell lines (Figure 5B). However, immunoblotting also shows that the total expression of β_1 integrin was also significantly suppressed

suggesting that the lower amounts of this integrin may be efficiently localized to the cell surface.

Preincubation with blocking antibodies against α_2 or β_1 integrin chains significantly inhibited the binding of MDA MB 231 cells, as expected (Figure 5C). The binding of one of the MDA MB 231/TM1 cells which showed a 50% reduction in binding to collagen (compared to the parental cells) was further reduced by the blocking antibodies. The attachment of the second cell line, which binds to collagen I very poorly, however was not further inhibited by the blocking antibodies.

We have also determined the effect of the blocking antibodies on binding the cells to polyL lysine as a negative control, and found that the inhibitory antibodies did not alter the binding of the cells to polyL lysine-coated dishes. Further, activation of integrins with the stimulatory antibody (TS2/16) enhanced binding of MDA MB 231 cells to collagen by about 40%, but did not rescue the diminished binding of MDA MB 231/TM1 cells to collagen I (data not shown). Together, these results indicate that TM1 expression induces a strong down regulation of coll receptor $\alpha_2\beta_1$ that results in a low binding to collagen.

Since MDA MB 231/TM1 cells bind to laminin more efficiently than the parental cells but have a down regulation of β1 integrin expression we examined the expression level of $\alpha_6\beta_4$ integrin, one of the major laminin receptors. MDA MB 231 cells express readily detectable amounts of α_6 and β_4 chains (Figure 6A). Surprisingly, in MDA MB 231/TM1 cells the expression of α_6 and β_4 integrins is lower. This suggests that either the residual α_6 and β_4 integrins effectively mediate binding to laminin or other integrins may mediate adhesion of MDA MB 231/TM1 cells to laminin.

To distinguish between these possibilities, we have pre-incubated the cells with function-blocking antibodies and tested for adhesion to laminin. The poor binding of MDA MB 231 cells to laminin, was not further inhibited by the inhibitory antibodies against α_6 and β_4 integrins (Figure 6B). However, the blocking antibodies against α_6 and β_4 integrins reduced binding of MDA MB 231/TM1 cells to laminin by 50%, indicating that the low levels of the $\alpha 6\beta 4$ integrin is highly active in mediating adhesion to laminin and indicating a possible involvement of other laminin-binding integrins in this event.

Because of the downregulation of $\alpha_{1-3,6}$ integrins in MDA MB 231/TM1 cells (Figure 5; and data not shown), it is likely that MDA MB 231/TM1 cells may utilize other integrins (e.g., $\alpha_9\beta 1$ integrin (49)) as a laminin receptor. Such studies are in progress.

Role of TM1-induced cytoskeleton in adhesion to ECM: MDA MB 231 cells lack stress fibers (Figure 1C), whereas the re-expression of TM1 promotes the formation of stress fibers on collagen and laminin (Figure 4D). We wished to determine the role of TM1-induced cytoskeleton in modulating binding to ECM.

The parental and MDA MB 231/TM1 cells were treated with Y-27632, a specific and widely employed inhibitor of Rho kinase (50) which mediates RhoA-driven stress fiber formation and the assembly of focal adhesions (51,52). Pretreatment of MDA MB 231 cells with the Rho kinase inhibitor significantly enhanced the binding of parental and vector control MDA MB 231 cells to collagen I (Figure 7A). The inhibition of Rho kinase enhanced cell spreading, formation of lamellipodia and actin polymerization (Figure 7B, left panel). In contrast, pharmacological inhibition of Rho kinase further decreased the binding of MDA MB 231/TM1 cells to collagen I (Figure 7A), severely

disrupted the microfilament organization and resulted in the formation of patches of F-actin (Figure 7A, right panel). Thus, malignant breast cancer cells appear to bind more efficiently and undergo cytoskeletal rearrangements on collagen when Rho kinase is inhibited, whereas the cells transduced with TM1 are dependent on Rho kinase activity for maximal binding to the ECM and stress fiber formation. Inhibition of Rho kinase during adhesion to laminin also produced a decreased binding in MDA MB 231/TM1 cells, although to a lesser extent, and in disruption of microfilaments (data not shown). Together, these results show that cells expressing TM1 are dependent on Rho kinase signaling for stress fiber assembly and adhesion to ECM.

Cytoskeletal disruption rescues breast cancer cells from TM1-induced anoikis: The altered cytoskeleton in tumor cell contributes to several cellular functions, and may also promote resistance to anoikis. One of the most prominent consequences of restoration of TM1 in malignant cells is the reorganization of actin microfilaments. While detachment of cells from the substratum results in the disorganization of prominent cytoskeletal structures such as stress fibers, it is likely that the residual cytoskeleton may be effective in inducing anoikis. To investigate the importance of TM1-induced cytoskeleton in re-sensitizing breast cancer cells to anoikis, we tested whether disruption of actin cytoskeleton would rescue cells from anoikis.

We have utilized Y-27632 and latrunculin A (LatA) to disrupt cytoskeleton and tested whether MDA MB 231/TM1 cells can be rescued from anoikis. Addition of either of the drugs resulted in a near 50% decrease in the DNA content in the subG_0-G_1 fraction of cell cycle, suggesting a role for cytoskeleton in sensitizing tumor cells to anoikis (Figure 8). The presence of drugs did not influence the anoikis resistance of parental

breast cancer cells, and the cells remained resistant to anoikis. These results suggest that TM1-induced cytoskeletal changes are involved in inducing anoikis in breast cancer cells.

Discussion:

Resistance to anoikis is an important mechanism acquired early on during neoplastic transformation that facilitates the survival of tumor cells in the absence of normal adhesion-dependent signaling. Therefore, mechanisms that trigger anoikis in tumor cells are of potential interest in designing anti-tumor therapies. Key findings of this study are that 1) TM1 promotes stress fiber assembly in breast carcinoma cells, and TM1-induced cytoskeletal reorganization modulates the activities of integrins and 2) re-sensitizes the tumor cells to anoikis. Thus, this work demonstrates the important role of microfilament-associated proteins and actin cytoskeletal organization in controlling the sensitivity to anoikis.

The sensitivity to anoikis is modulated through inhibition of intracellular signaling pathways controlled by extracellular signal regulated kinase (ERK) or Protein kinase B/AKT signaling, and modulation of the activities of integrins. Resensitization of tumor cells to anoikis has been shown to occur via death receptor mediated extrinsic pathways of apoptosis (47,53,54). However, anoikis of mammary epithelial cells progresses through the intrinsic pathways of apoptosis through cytochrome C release from mitochondria (46). TM1-mediated anoikis is also dependent on caspase activation and occurs through the intrinsic pathways of cell death.

Based on these biochemical functions of TMs, we propose that TM1 exerts its anti-oncogenic effects through reorganization of cytoskeleton (Figure 9). According to this model, TM1 promotes stress fibers assembly and the reorganization of cytoskeleton

modulates intracellular signaling pathways leading to altered cell-ECM interactions and consequently adhesion-dependency is restored for cell survival.

Role of cytoskeleton in TM1-induced anoikis: It is intriguing that a structural protein, like TM1, with no known catalytic activity, exerts a profound effect on cell growth. Although some studies indicate that TMs binds to other non-cytoskeletal regulatory proteins (55), it is unclear that such interactions are important for TM1 functions. Therefore, the antioncogenic functions of TM1 and other cytoskeletal proteins may be dependent on their cytoskeletal effects. Further, TM isoforms(31,34) or variants of TM1 (40) that fail to induce cytoskeletal changes do not function as suppressors of neoplastic growth. TM1, in conjunction with caldesmon, primarily stabilizes actin microfilaments against the action of gel severing proteins such as gelsolin and cofilin. Second, TMs regulate actin-myosin interactions, inhibit acto-myosin ATPase activity and modulate cellular contractility. It has been suggested that the downregulation of cytoskeletal proteins in neoplastic transformation may promote cellular contractility and destabilize focal adhesions, whereas inhibition of cellular contractility in ras-transformed cells results in a partial revertant phenotype.

Whereas it is well established that reorganization of actin cytoskeleton occurs upon activation of adhesion-dependent signaling (8), it is not well understood if the cytoskeletal changes are important in resistance to anoikis. Rac and Cdc42 which primarily control the assembly of lamellipodia and filopdia, respectively, are reported to confer resistance to anoikis (56,57). It remains to be determined whether RhoA, which in normal cells controls the assembly of stress fibers and focal adhesions, has any role in anoikis. However, in malignant cells RhoA has been suggested to be uncoupled from its

downstream effectors which may explain the lack of stress fibers in transformed cells (58-60). Data presented here (Figures 7 and 8) and elsewhere (37) demonstrate that TM1-induced cytoskeletal changes are mediated through Rho-kinase regulated pathways, and that Rho kinase signaling may be important in TM1-induced anoikis.

We have hypothesized that the cytoskeletal reorganization by TM1 as the key to induction of anoikis (Figure 9). The cytoskeletal disrupting drugs, such as LatA, induce apoptosis in normal MCF10A cells (29). In our hands treatment of MDA MB 231/TM1 cells with a much reduced amounts of Lat A (0.5μM) disrupted the cytoskeletal organization, abolished binding to collagen I in adherent cells. However, disruption of cytoskeleton with LatA in suspension rescues cells from anoikis, further underscoring the role of actin cytoskeleton in cell survival.

Although adhesion-deprived cells lack extensive cytoskeletal structures like stress fibers, they may contain residual cytoskeleton. Both LatA and the Y compound rescue breast cancer cells from TM1-induced anoikis. Thus, TM1 may induce anoikis through cytoskeletal changes controlled by Rho kinase. Recent work from this laboratory shows that TM1 may inhibit the activity and limit the access of cofilin to actin cytoskeleton and thus protect the cytoskeleton in a Rho kinase dependent fashion (40). Thus, the ability of TM1 to promote cytoskeleton is critical for its effects.

Role of integrins in TM1-induced anoikis: Integrins and microfilaments have been proposed to engage in bidirectional signaling and regulate survival, cell growth and differentiation (61). While it is well established that integrin activation reorganizes microfilaments (reviewed in (8)), the present work shows that integrin activity can be modulated by changes in cytoskeletal organization. Other key cytoskeletal proteins, such

as vinculin and talin, interact with F-actin and the cytoplasmic tails of integrins at the focal adhesions, and thus regulate integrin activity, cytoskeletal reorganization and signaling pathways (62,63). However, TMs bind to F-actin but are not known to associate with focal adhesions. Therefore, we suggest that TM1-induced cytoskeletal changes may regulate intracellular signaling, integrin expression and activity, and cell survival (Figure 9).

Neoplastic cells exhibit altered integrin expression and activation which may result in the constitutive activation of cell survival pathways independent of ECM-mediated integrin activation. TM1 induces anoikis in both serum free (31) and serum containing media (Table 1), indicating that TM1 restores the requirement of adhesion-dependent signaling through modulation of the activity and/or expression of integrins. Such a mechanism is consistent with the previous observations which suggest that integrins as key modulators of anoikis (17-19). Investigation into the role of integrins revealed that TM1 expression inhibits the activity of collagen I-binding integrins and upregulation of the activity of laminin receptors. The downregulation of $\alpha_2\beta_1$ integrin, a major collagen receptor, may result in the poor binding to collagen I. We find that TM1 expression profoundly downregulates the expression of α_2 integrin. Conversely, the expression of α_2 integrin is enhanced in Raf-induced resistance to anoikis of mammary epithelial cells (64). Further, Src-induced cell adhesion of breast cancer is reported to be mediated through α_2 integrin (65). While we did not find significant changes in the surface expression of β_1 integrin, the total expression of this integrin is markedly reduced in TM1-transduced cells (Figure 5B), suggesting that the reduced β_1 integrin may be efficiently localized to cell surface and may associate with a different α integrin.

Consistent with the downregulation of α_2, addition of β_1 activating antibodies (TS2/16) did not enhance binding to collagen or enhance survival of MDA MB 231/TM1 cells (data not shown). At present it is unclear whether downregulation of $\alpha_2\beta_1$ integrin contributes to TM1-induced anoikis. Although enhanced expression of this integrin is observed in anoikis resistance (64) and shown to be critical in mammary carcinogenesis (66), the expression of this integrin is downregulated in many breast tumors (67). While downregulation of $\alpha_2\beta_1$ integrin in TM1-transduced cells explains the decreased binding to collagen I, its significance in TM1-induced anoikis and the contribution of the altered expression and activity of this integrin may require further study.

Notwithstanding the increased binding to laminin, MDA MB 231/TM1 cells, these cells contained significantly reduced amounts of the laminin binding $\alpha_6\beta_4$ integrin. The $\alpha_6\beta_4$ integrin has been shown to be an important determinant in regulating tumor cell survival and motility (reviewed in (68,69)). Currently, work is in progress to determine whether other integrins (e.g., $\alpha_9\beta1$) promote TM1-mediated enhancement of binding to laminin. At present we can not exclude the participation of other integrins in TM1-induced anoikis. For example, TM1 transduced cells, like the parental MDA MB 231 cells, express readily detectable amounts of α_3 and β_5 integrins (data not shown) which may regulate anoikis. While we find specific changes in the integrin profile and activity following TM1 expression, the association of TM1-induced cytoskeleton with integrins may be critical to adhesion-dependent signaling. The interactions between TM1-induced cytoskeleton and the focal adhesion molecules may control cell survival in adhesion-dependent fashion.

A key question that remains to be addressed in this model (Figure 9) is how does the cytoskeleton regulate the expression and the activities of integrins? The cytoskeleton may anchor the key signaling enzymes or their substrates (70-72), regulate the subcellular distribution of the enzymes or the polymerization status of actin itself may act as a regulator of gene expression (70,71,73-75). Elucidation of the mechanisms of that drive the changes in gene expression following the expression of TM1 in breast cancer cells are under investigation.

In summary, we report that restoration of TM1 expression promotes stress fiber assembly, alters integrin expression and activity and induces anoikis through intrinsic pathways of apoptosis.

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References:

1. Schmeichel, K. L., Weaver, V. M., and Bissell, M. J. (1998) *J Mammary Gland Biol Neoplasia* **3**, 201-213
2. Chrenek, M. A., Wong, P., and Weaver, V. M. (2001) *Breast Cancer Res* **3**, 224-229
3. Bissell, M. J., and Radisky, D. (2001) *Nat Rev Cancer* **1**, 46-54
4. Shekhar, M. P., Pauley, R., and Heppner, G. (2003) *Breast Cancer Res* **5**, 130-135
5. Haslam, S. Z., and Woodward, T. L. (2003) *Breast Cancer Res* **5**, 208-215
6. Frisch, S. M., and Screaton, R. A. (2001) *Current Opinion in Cell Biology* **13**, 555-562
7. Schwartz, M. A. (1997) *Journal of Cell Biology* **139**, 575-578
8. DeMali, K. A., Wennerberg, K., and Burridge, K. (2003) *Current Opinion in Cell Biology* **15**, 572-582
9. Aplin, A. E., and Juliano, R. L. (2001) *J Cell Biol* **155**, 187-191
10. Debnath, J., Mills, K. R., Collins, N. L., Reginato, M. J., Muthuswamy, S. K., and Brugge, J. S. (2002) *Cell* **111**, 29-40
11. Debnath, J., Muthuswamy, S. K., and Brugge, J. S. (2003) *Methods* **30**, 256-268
12. Manohar, A., Shome, S. G., Lamar, J., Stirling, L., Iyer, V., Pumiglia, K., and DiPersio, C. M. (2004) *J Cell Sci* **117**, 4043-4054
13. Swan, E. A., Jasser, S. A., Holsinger, F. C., Doan, D., Bucana, C., and Myers, J. N. (2003) *Oral Oncology* **39**, 648-655
14. Coll, M. L., Rosen, K., Ladeda, V., and Filmus, J. (2002) *Oncogene* **21**, 2908-2913

15. Rosen, K., Coll, M. L., Li, A., and Filmus, J. (2001) *J Biol Chem* **276**, 37273-37279

16. Normanno, N., Luca, A. D., Bianco, C., Maiello, M. R., Carriero, M. V., Rehman, A., Wechselberger, C., Arra, C., Strizzi, L., Sanicola, M., and Salomon, D. S. (2004) *J Cell Physiol* **198**, 31-39

17. Strater, J., Wedding, U., Barth, T. F., Koretz, K., Elsing, C., and Moller, P. (1996) *Gastroenterology* **110**, 1776-1784

18. Kozlova, N. I., Morozevich, G. E., Chubukina, A. N., and Berman, A. E. (2001) *Oncogene* **20**, 4710-4717

19. Janes, S. M., and Watt, F. M. (2004) *J. Cell Biol.* **166**, 419-431

20. Davies, M. A., Lu, Y., Sano, T., Fang, X., Tang, P., LaPushin, R., Koul, D., Bookstein, R., Stokoe, D., Yung, W. K., Mills, G. B., and Steck, P. A. (1998) *Cancer Res* **58**, 5285-5290

21. Lu, Y., Lin, Y. Z., LaPushin, R., Cuevas, B., Fang, X., Yu, S. X., Davies, M. A., Khan, H., Furui, T., Mao, M., Zinner, R., Hung, M. C., Steck, P., Siminovitch, K., and Mills, G. B. (1999) *Oncogene* **18**, 7034-7045

22. Plath, T., Detjen, K., Welzel, M., von Marschall, Z., Murphy, D., Schirner, M., Wiedenmann, B., and Rosewicz, S. (2000) *J Cell Biol* **150**, 1467-1478

23. Rocco, J. W., and Sidransky, D. (2001) *Exp Cell Res* **264**, 42-55

24. Lin, J. J., Warren, K. S., Wamboldt, D. D., Wang, T., and Lin, J. L. (1997) *International Review of Cytology* **170**, 1-38

25. Pawlak, G., and Helfman, D. M. (2001) *Current Opinion in Genetics & Development* **11**, 41-47

26. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) *Science* **302**, 1704-1709

27. Suetsugu, S., and Takenawa, T. (2003) *Int Rev Cytol* **229**, 245-286

28. Raftopoulou, M., and Hall, A. (2004) *Dev Biol* **265**, 23-32

29. Martin, S. S., and Leder, P. (2001) *Molecular & Cellular Biology* **21**, 6529-6536

30. Bhattacharya, B., Prasad, G. L., Valverius, E. M., Salomon, D. S., and Cooper, H. L. (1990) *Cancer Research* **50**, 2105-2112

31. Raval, G. N., Bharadwaj, S., Levine, E. A., Willingham, M. C., Geary, R. L., Kute, T., and Prasad, G. L. (2003) *Oncogene* **22**, 6194-6203

32. Pawlak, G., McGarvey, T. W., Nguyen, T. B., Tomaszewski, J. E., Puthiyaveettil, R., Malkowicz, S. B., and Helfman, D. M. (2004) *Int J Cancer* **110**, 368-373

33. Prasad, G. L., Fuldner, R. A., and Cooper, H. L. (1993) *Proceedings of the National Academy of Sciences of the United States of America* **90**, 7039-7043

34. Braverman, R. H., Cooper, H. L., Lee, H. S., and Prasad, G. L. (1996) *Oncogene* **13**, 537-545

35. Prasad, G. L., Masuelli, L., Raj, M. H., and Harindranath, N. (1999) *Oncogene* **18**, 2027-2031

36. Mahadev, K., Raval, G., Bharadwaj, S., Willingham, M. C., Lange, E. M., Vonderhaar, B. K. V., Salomon, D., and Prasad, G. L. (2002) *Experimental Cell Research.* **279**, 40-51

37. Shah, V., Bharadwaj, S., Kaibuchi, K., and Prasad, G. L. (2001) *Oncogene* **20**, 2112-2121

38. Cooper, J. A. (2002) *Curr Biol* **12**, R523-525

39. Marston, S., Burton, D., Copeland, O., Fraser, I., Gao, Y., Hodgkinson, J., Huber, P., Levine, B., el-Mezgueldi, M., and Notarianni, G. (1998) *Acta Physiol Scand* **164**, 401-414

40. Bharadwaj, S., Hitchcock-DeGregori, S., Thorburn, A., and Prasad, G. L. (2004) *J. Biol. Chem.*, M310934200

41. Falcioni, R., Sacchi, A., Resau, J., and Kennel, S. J. (1988) *Cancer Res* **48**, 816-821

42. Maemura, M., Akiyama, S. K., Woods, V. L., Jr., and Dickson, R. B. (1995) *Clinical & Experimental Metastasis* **13**, 223-235

43. Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., and Borner, C. (1998) *Nature* **391**, 496-499

44. Bharadwaj, S., and Prasad, G. L. (2002) *Cancer Letters* **183**, 205-213.

45. Aoudjit, F., and Vuori, K. (2001) *Journal of Cell Biology* **152**, 633-643

46. Valentijn, A. J., Metcalfe, A. D., Kott, J., Streuli, C. H., and Gilmore, A. P. (2003) *J Cell Biol* **162**, 599-612

47. Stupack, D. G., and Cheresh, D. A. (2002) *J Cell Sci* **115**, 3729-3738

48. Heino, J. (2000) *Matrix Biology* **19**, 319-323

49. Palmer, E., Ruegg, C., Ferrando, R., Pytela, R., and Sheppard, D. (1993) *J. Cell Biol.* **123**, 1289-1297

50. Narumiya, S., Ishizaki, T., and Uehata, M. (2000) *Methods Enzymol* **325**, 273-284

51. Chrzanowska-Wodnicka, M., and Burridge, K. (1996) *Journal of Cell Biology* **133**, 1403-1415

52. Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997) *Science* **275**, 1308-1311

53. Valentijn, A. J., Zouq, N., and Gilmore, A. P. (2004) *Biochem Soc Trans* **32**, 421-425

54. Martin, S. S., and Vuori, K. (2004) *Biochim Biophys Acta* **1692**, 145-157

55. Li, Q., Dai, Y., Guo, L., Liu, Y., Hao, C., Wu, G., Basora, N., Michalak, M., and Chen, X. Z. (2003) *J Mol Biol* **325**, 949-962

56. Coniglio, S. J., Jou, T. S., and Symons, M. (2001) *J Biol Chem* **276**, 28113-28120

57. Cheng, T.-L., Symons, M., and Jou, T.-S. (2004) *Experimental Cell Research* **295**, 497-511

58. Izawa, I., Amano, M., Chihara, K., Yamamoto, T., and Kaibuchi, K. (1998) *Oncogene* **17**, 2863-2871

59. Sahai, E., Olson, M. F., and Marshall, C. J. (2001) *EMBO J.* **20**, 755-766

60. Pawlak, G., and Helfman, D. M. (2002) *Mol Biol Cell* **13**, 336-347

61. Schoenwaelder, S. M., and Burridge, K. (1999) *Current Opinion in Cell Biology* **11**, 274-286

62. Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2000) *Journal of Biological Chemistry* **275**, 22607-22610

63. Critchley, D. R. (2000) *Curr Opin Cell Biol* **12**, 133-139

64. Schulze, A., Lehmann, K., Jefferies, H. B., McMahon, M., and Downward, J. (2001) *Genes & Development* **15**, 981-994

65. Park, H. B., Golubovskaya, V., Xu, L., Yang, X., Lee, J. W., Scully, S., 2nd, Craven, R. J., and Cance, W. G. (2004) *Biochem J* **378**, 559-567

66. White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U., and Muller, W. J. (2004) *Cancer Cell* **6**, 159-170

67. Alford, D., Pitha-Rowe, P., and Taylor-Papadimitriou, J. (1998) *Biochem Soc Symp* **63**, 245-259

68. Mercurio, A. M., Rabinovitz, I., and Shaw, L. M. (2001) *Current Opinion in Cell Biology* **13**, 541-545

69. Chung, J., Yoon, S., Datta, K., Bachelder, R. E., and Mercurio, A. M. (2004) *Cancer Res* **64**, 4711-4716

70. Fincham, V. J., James, M., Frame, M. C., and Winder, S. J. (2000) *EMBO J.* **19**, 2911-2923

71. Woodring, P. J., Hunter, T., and Wang, J. Y. (2001) *J Biol Chem* **276**, 27104-27110

72. Christerson, L. B., Vanderbilt, C. A., and Cobb, M. H. (1999) *Cell Motil Cytoskeleton* **43**, 186-198

73. Woodring, P. J., Litwack, E. D., O'Leary, D. D., Lucero, G. R., Wang, J. Y., and Hunter, T. (2002) *J Cell Biol* **156**, 879-892

74. Aplin, A. E., Stewart, S. A., Assoian, R. K., and Juliano, R. L. (2001) *J Cell Biol* **153**, 273-282

75. Geneste, O., Copeland, J. W., and Treisman, R. (2002) *J. Cell Biol.* **157**, 831-838

Figure Legends:

Figure 1. Restoration of TM1 expression alters cellular morphology and promotes stress fiber assembly: (A). MDA MB 231 cells were retrovrially transduced with TM1 and the isolated cell lines were tested for TM1 expression using a TM1-specific antibody. The parental cells are negative for TM1 expression whereas two independent clones are positive. The blot was probed with tubulin antibody for load controls. (B). Light microscopic examination (20x magnification shown) reveals that the parental cells display spindle shaped morphology, while MDA MB 231/TM1 cells grow as tight clusters with increased cell spreading. (C). TM1 expression results in the reorganization of microfilaments and promotes stress fiber assembly as determined by immunostaining with TM polyclonal antibody (green) and Texas-Red conjugated phalloidin followed by confocal microscopy. MDA MB 231 cells lack stress fibers.

Figure 2. TM1 induces detachment induced apoptosis in MDA MB 231 cells: (A). MDA MB 231/TM1 cells transduced with TM1, cultured in suspension in regular growth medium for 24h, cytospun and stained with DAPI. Bright field images of the same field are also shown. In contrast to the parental cells, TM1-transduced cells contain fragmented nuclear DNA. The percentage of cells undergoing apoptosis (mean ± s.d.) is depicted in (B). The MDA MB 231/TM1 cells (two cell lines) undergo apoptosis in suspension cultures (sus), while the adherent (adh) cultures undergo background apoptosis. TM1-induced anoikis is detectable by 6h of suspension culture (data not shown) and a majority of the cells are apoptotic by 24h. (C). The adherent and suspension cultures were stained with annexin V, a marker of apoptosis, and the relative

fluorescent intensities (mean ± s.d.) are shown. MDA MB 231/TM1 cells in suspension culture for 24h showed significantly higher annexin staining, indicating cell death. (D). Cell lysates from MDA MB 231 and TM1-transduced cells cultured as monolayers (indicated as 0), or in suspension for 6h or 24h (lanes shown as 6 and 24) were analyzed for PARP cleavage. The monolayer cells were treated with etoposide (lanes marked ET) and tested for PARP cleavage. The drug treated samples and MDA MB 231/TM1 cells in suspension contain significantly higher amounts of cleaved PARP compared to MDA MB 231 cells in suspension.

Figure 3. TM1-induced anoikis is caspase dependent and progresses through the intrinsic pathway of apoptosis: (A). TM1-induced anoikis is mediated through the activation of caspases 3 and 7. Immunoblotting of cell lysates from untreated adherent (0), etoposide (ET)-treated, or suspension culture (6 and 24h) of MDA MB 231, vector control (MDA MB 231/V) and two cell lines of MDA MB 231/TM1 cells were probed with antibodies against caspase 3 and 7. The presence of cleaved products indicates the activation of respective caspases. (B). Immunofluorescence images of cells stained with cytochrome C. Suspension cultures (6 and 24h) were detergent extracted and stained with antibodies against cytochrome C and the images were recorded by confocal microscopy. (C). MDA MB 231/TM1 cells were either untreated (control), or treated with DMSO or ZVAD for 24h in suspension cultures along with untreated MDA MB 231 cells. The cell lysates were analyzed for activation of caspases 3 and 7 by immunoblotting. (D). Anoikis by control (open bars) and ZVAD-treated (filled bars) cells was tested by measuring the

fraction of DNA in subG_0-G_1 portion of cell cycle and the mean ± s.d. were plotted. Addition of ZVAD blocks TM1-induced anoikis.

Figure 4. TM1 modulates integrin activity and alters interactions with ECM: (A). Parental, vector control and TM1-transduced MDA MB 231 cells were plated on collagen I (col I), fibronectin (FN), laminin (Lam) or poly L lysine (poly L)-coated dishes in triplicate for 30 minutes and the cell adhesion was measured by crystal violet staining (42). The binding of MDA MB 231 cells to each ECM component (or poly L lysine) was taken as 100%. The error bars represent standard deviation (n=3). (B). TM1 expression inhibits cell adhesion to collagen I, but not collagen IV. The cell lines were plated on ECM components as indicated in the inset. (C). Light microscopic images of cells plated on ECM components for 30 minutes and stained with crystal violet (40x) magnification. (D). The indicated cells were plated on collagen I (top panels) and laminin (bottom panels) for 30 minutes and stained with phalloidin. Confocal microscopic images are shown.

Figure 5. Downregulation of integrins in TM1-transduced breast carcinoma cells: (A). The cell surface expression of $\alpha_2\beta_1$ was determined by flow cytometry as described in Methods. (B). Total cellular expression of $\alpha_2$and β_1 was determined by immunoblotting from 2% SDS solubilized actively growing cultures. (C). The binding of MDA MB 231 and MDA MB 231/TM1 cells to collagen I was tested in cell adhesion assay (n=3) using the inhibitory antibodies against α_2 and β_1 integrins. The binding of MDA MB 231 cells

in the absence of the inhibitory antibodies is taken as 100%. The mean and standard deviation are plotted.

Figure 6. TM1 expression inhibits $\alpha_6\beta_4$ integrin activity: (A). The expression of $\alpha_6\beta_4$ integrin was determined by flow cytometry and the relative fluorescent intensities obtained with each cell line is depicted. (B). The binding of MDA MB 231 and MDA MB 231/TM1 cells to laminin was tested in cell adhesion assay (n=3) using the inhibitory antibodies against α_6 and β_4 integrin chains. The binding of MDA MB 231 cells in the absence of the inhibitory antibodies is taken as 100%, and the mean and standard deviation are plotted.

Figure 7. Attachment of TM1-expressing cells, but not the parental MDA MB 231 cells, to collagen requires Rho kinase signaling: (A). Cell adhesion (30 minutes) of indicated cell lines in presence or absence of Y27632. The binding of untreated MDA MB 231 cells is taken as 100%. (B). Cytoskeletal reorganization in presence of the Rho kinase inhibitor during cell adhesion. Confocal microscopic images of phalloidin stained cells are shown.

Figure 8. Rho kinase and cytoskeletal integrity are required for TM1-induced anoikis: Cells were cultured in suspension for 24h in the presence of Y27632, latrunculin A or untreated (control), and the anoikis was assayed by measuring the DNA content of subG0-G1 fraction of cell cycle by flow cytometry. Relative anoikis for each cell line

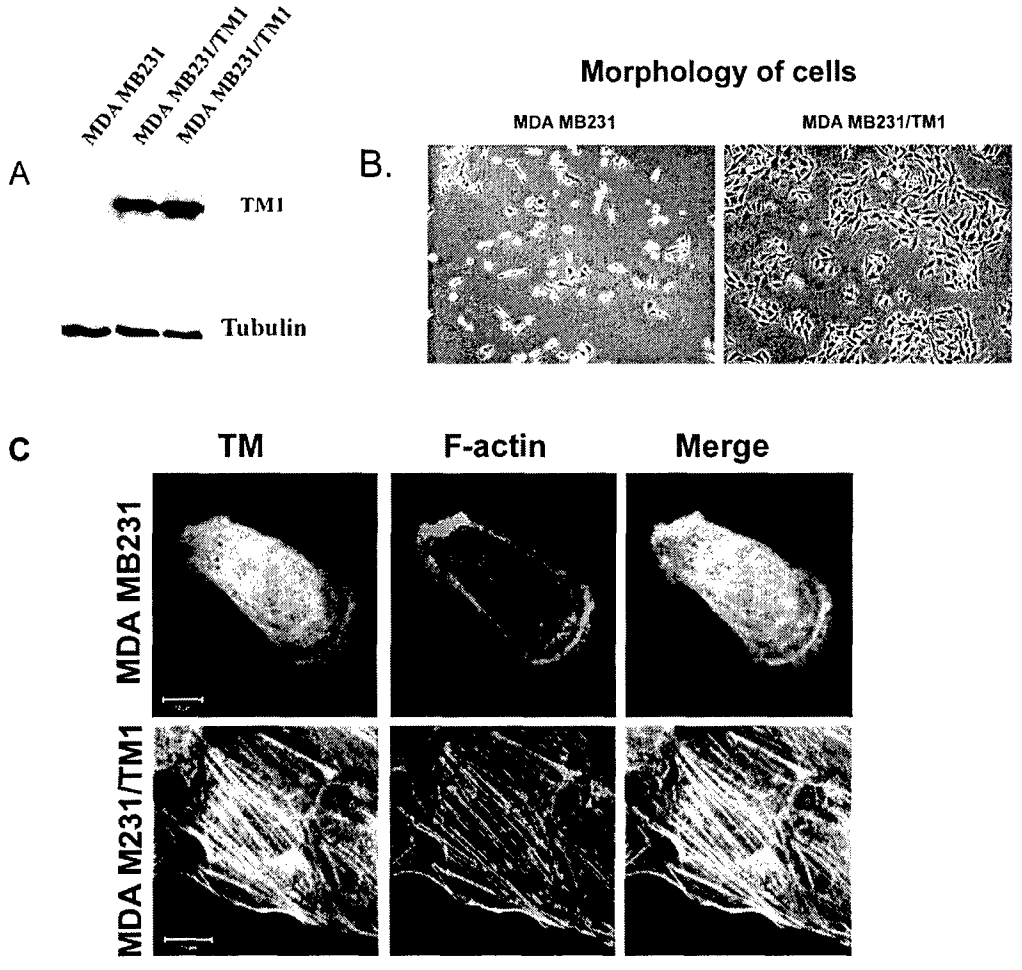
was calculated using the control value of 100%. Mean and standard deviation from 3 independent experiments is depicted.

Figure 9. *Hypothetical events in TM1-induced anoikis*: Most tumor cells contain aberrant cytoskeleton, constitutively active integrins and deregulated intracellular signaling resulting in an anoikis resistant phenotype (depicted with gray arrows). We propose that TM1 expression results in the reorganization of cytoskeleton with the assembly of stress fibers, leading to altered intracellular signaling pathways and integrin expression and activation. Thus the adhesion dependency on survival pathways is restored and the tumor cells are re-sensitized to anoikis (depicted with solid arrows).

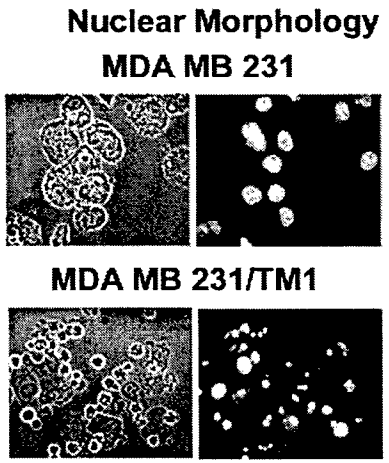
Table-I: Effect of serum on TM1-induced anoikis

Cell Line	6 hours in suspension			24 hours in suspension		
	No serum	1% Serum	10% Serum	No Serum	1% Serum	10% Serum
MDA-MB231	0.99±0.12	3.49±0.48	2.05±0.71	3.39±0.82	2.85±0.12	5.48±2.32
MDA-MB231/TM1	17.07±2.98	10.29±1.51	14.69±7.37	81.65±4.52	56.74±7.96	55.89±5.28
MDA-MB231/TM1	30.21±6.25	9.49±1.52	14.46±2.90	77.87±9.13	60.87±6.72	57.87±1.53

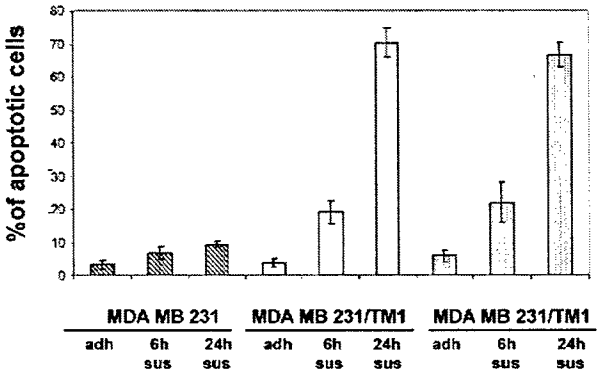
Cells were cultured for indicated times and the anoikis was determined by quantifying DNA in the subG_0-G_1 fraction of cell cycle.


A
MDA MB231
MDA MB231/TM1
MDA MB231/TM1
TM1
Tubulin
B.
Morphology of cells
MDA MB231
MDA MB231/TM1
C
TM
F-actin
Merge
MDA MB231
MDA M231/TM1

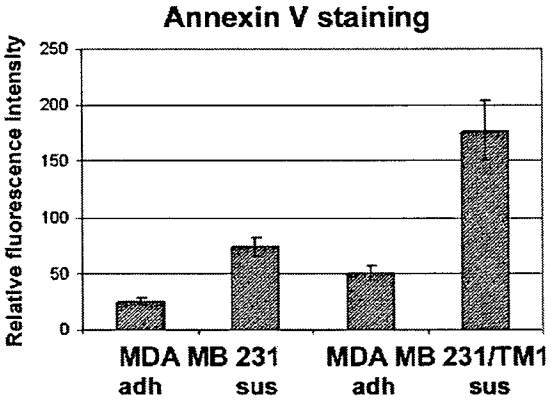
A.


Nuclear Morphology
MDA MB 231
MDA MB 231/TM1

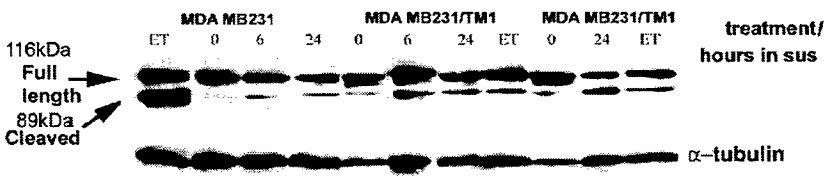
B.


%of apoptotic cells
0
10
20
30
40
50
60
70
80
MDA MB 231
MDA MB 231/TM1
MDA MB 231/TM1
adh
6h sus
24h sus
adh
6h sus
24h sus
adh
6h sus
24h sus

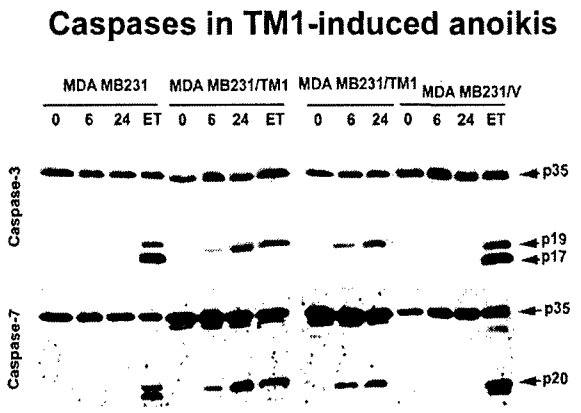
C.


Annexin V staining
Relative fluorescence intensity
0
50
100
150
200
250
MDA MB 231
adh
sus
MDA MB 231/TM1
adh
sus

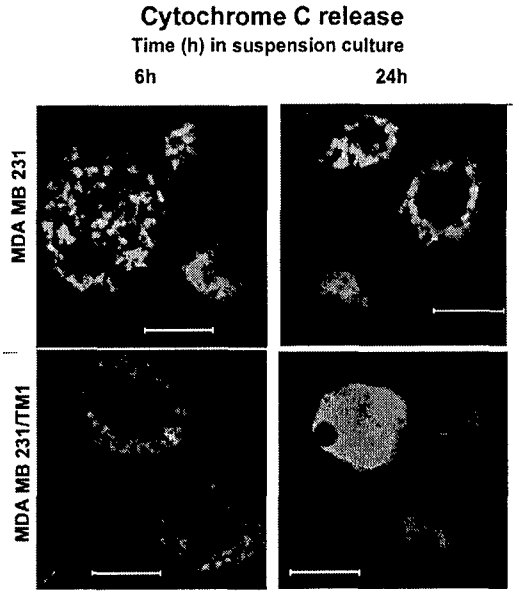
D.


Cleavage of PARP
MDA MB231
MDA MB231/TM1
MDA MB231/TM1
ET 0 6 24 0 6 24 ET 0 24 ET
treatment/
hours in sus
116kDa
Full
length
89kDa
Cleaved
α–tubulin

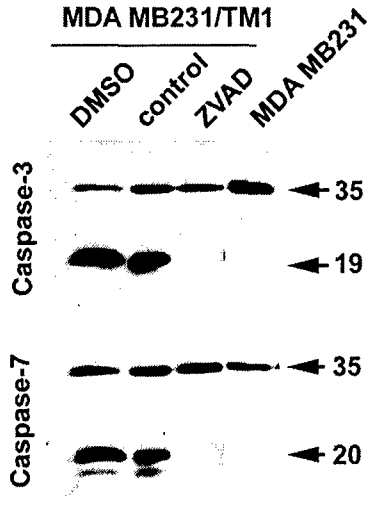
A.


Caspases in TM1-induced anoikis
MDA MB231
MDA MB231/TM1
MDA MB231/TM1
MDA MB231/V
0 6 24 ET 0 6 24 ET 0 6 24 0 6 24 ET
Caspase-3
Caspase-7
p35
p19
p17
p35
p20

B.


Cytochrome C release
Time (h) in suspension culture
6h
24h
MDA MB 231
MDA MB 231/TM1

C.


Inhibition of caspases
MDA MB231/TM1
DMSO
control
ZVAD
MDA MB231
Caspase-3
Caspase-7
35
19
35
20

D.

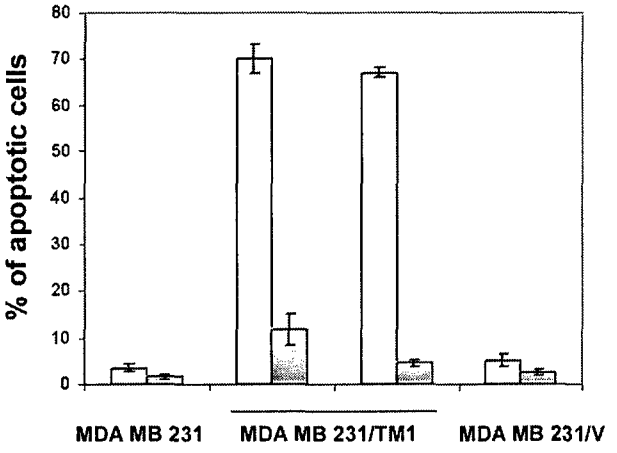

Inhibition of caspases rescues TM1-induced anoikis
% of apoptotic cells
80
70
60
50
40
30
20
10
0
MDA MB 231
MDA MB 231/TM1
MDA MB 231/V

Figure 4

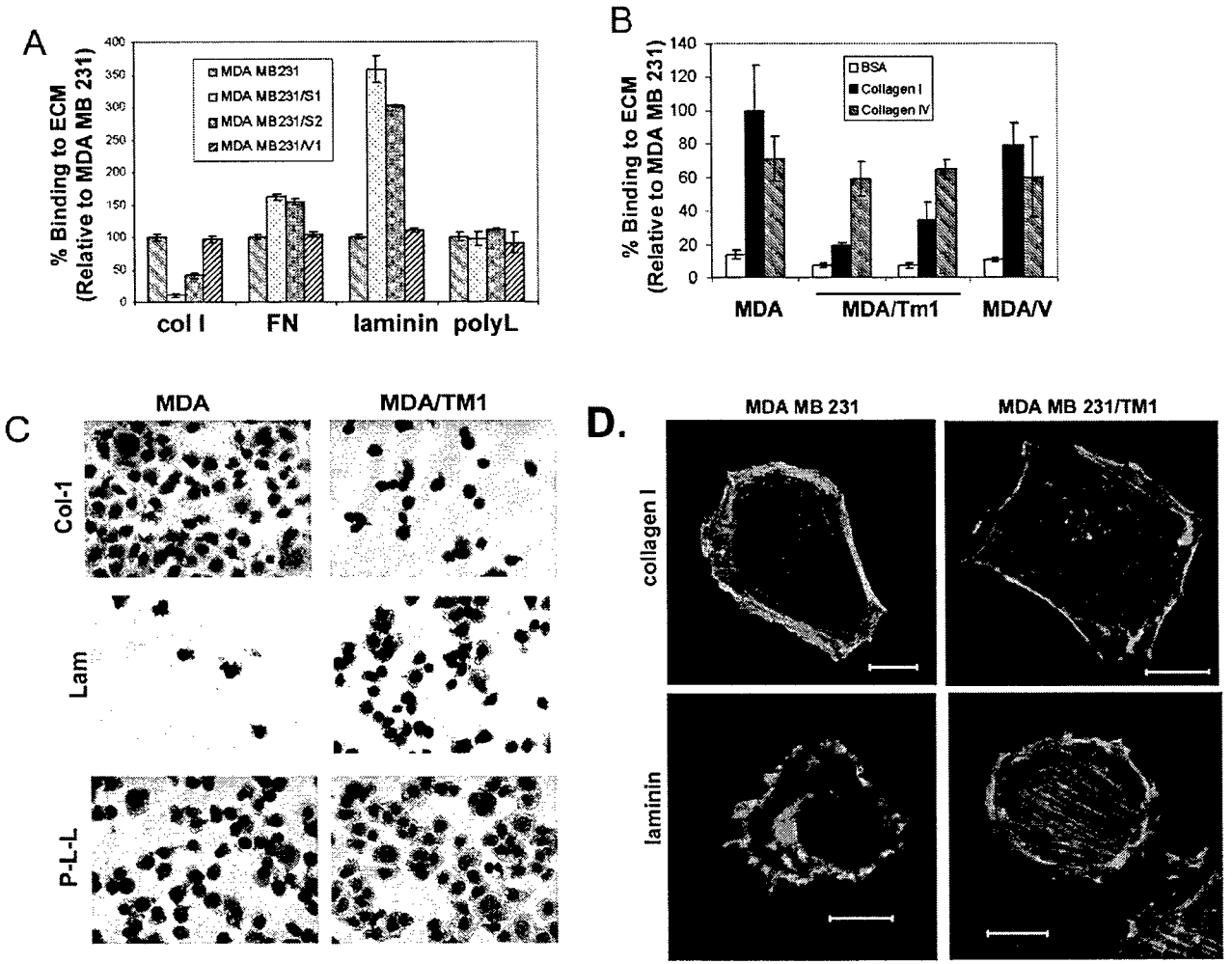

A
% Binding to ECM (Relative to MDA MB 231)
MDA MB231
MDA MB231/S1
MDA MB231/S2
MDA MB231/V1
col I
FN
laminin
polyL
B
% Binding to ECM (Relative to MDA MB 231)
BSA
Collagen I
Collagen IV
MDA
MDA/Tm1
MDA/V
C
MDA
MDA/TM1
Col-1
Lam
P-L-L
D.
MDA MB 231
MDA MB 231/TM1
collagen I
laminin

Figure 5

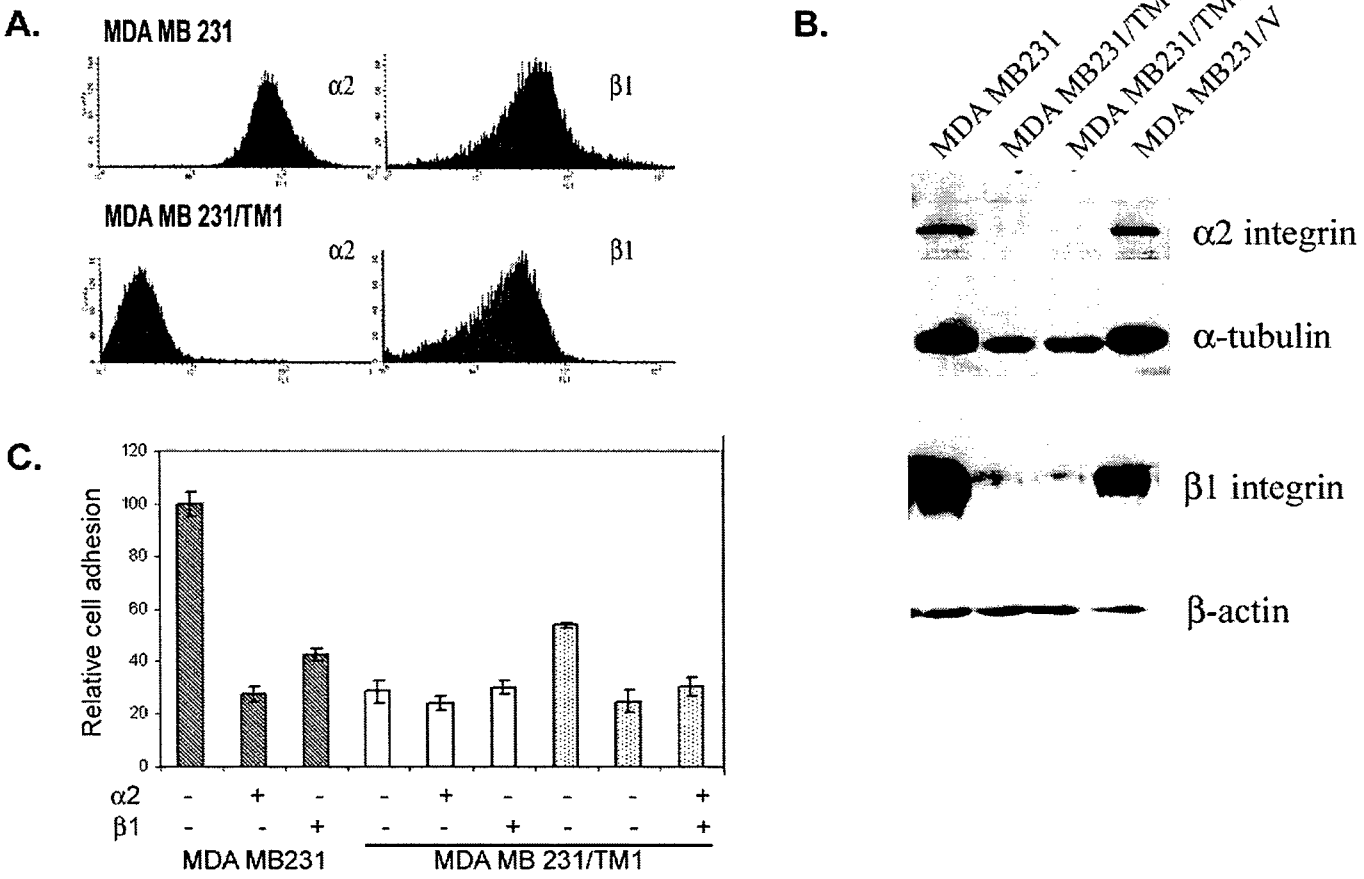
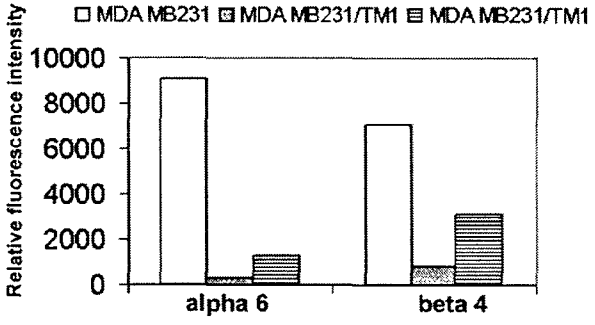

A.
MDA MB 231
α2
β1
MDA MB 231/TM1
α2
β1
B.
MDA MB231
MDA MB231/TM1
MDA MB231/TM1
MDA MB231/V
α2 integrin
α-tubulin
β1 integrin
β-actin
C.
Relative cell adhesion
120
100
80
60
40
20
0
α2 - + - - + - - - +
β1 - - + - - + - - +
MDA MB231
MDA MB 231/TM1

Figure 6

A.


MDA MB231
MDA MB231/TM1
MDA MB231/TM1
Relative fluorescence intensity
10000
8000
6000
4000
2000
0
alpha 6
beta 4

B.

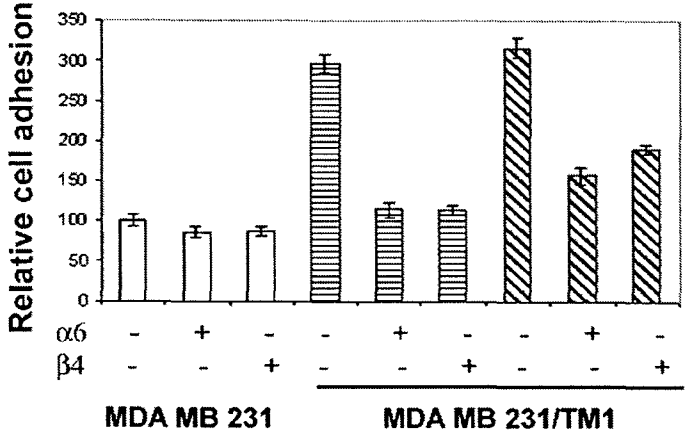
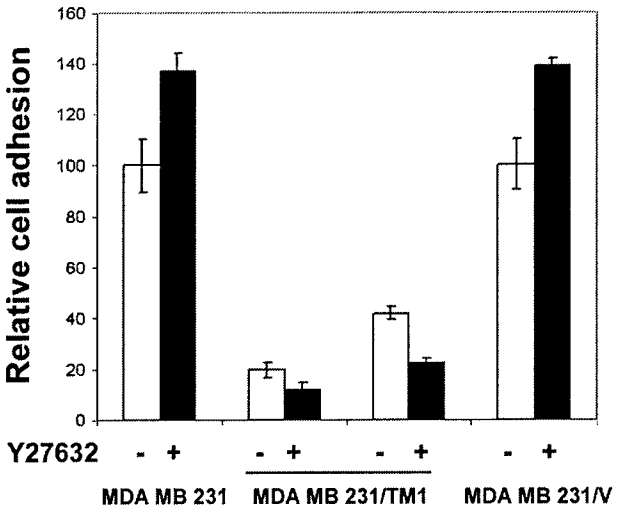

Relative cell adhesion
350
300
250
200
150
100
50
0
α6 - + - - + - - + -
β4 - - + - - + - - +
MDA MB 231
MDA MB 231/TM1

Figure 7

A.


Relative cell adhesion
160
140
120
100
80
60
40
20
0
Y27632 - + - + - + - +
MDA MB 231
MDA MB 231/TM1
MDA MB 231/V

B.

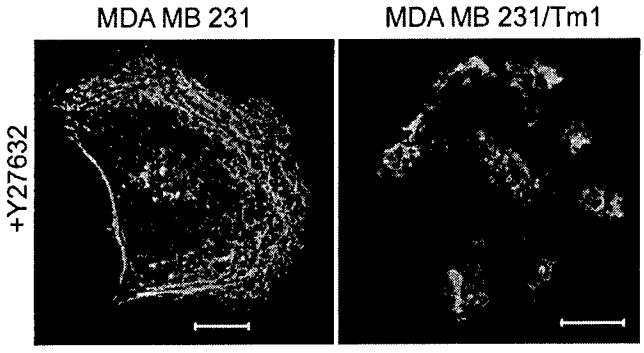

MDA MB 231
MDA MB 231/Tm1
+Y27632

Figure 8

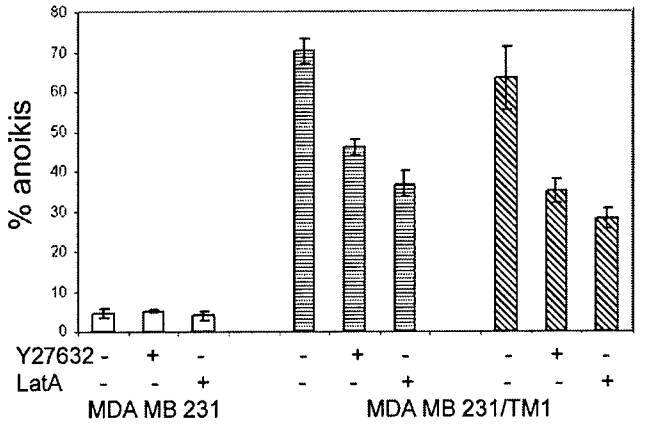
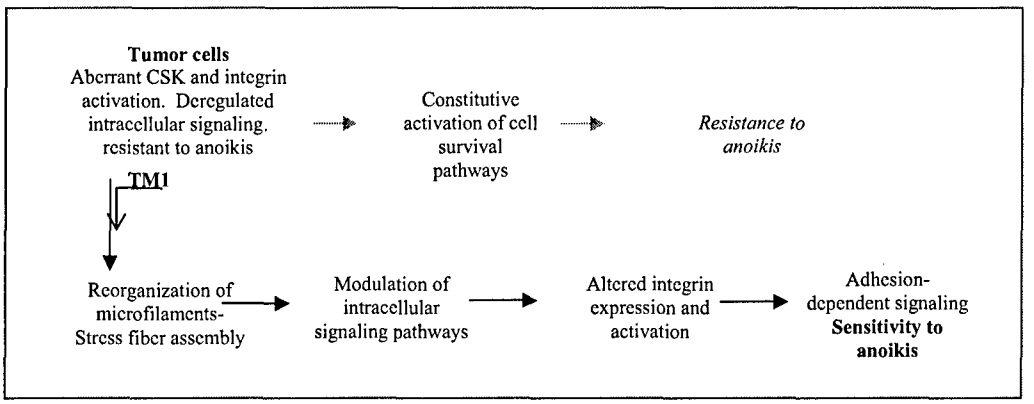

% anoikis
80
70
60
50
40
30
20
10
0
Y27632 - + - - + - - + -
LatA - - + - - + - - +
MDA MB 231
MDA MB 231/TM1

Figure 9: Hypothetical events in TM1-induced anoikis.



APPENDIX 7

manuscript under Consideration

Amino terminal, but not the carboxy terminal, sequences of Tropomyosin-1 are essential for the induction of stress fiber assembly in neoplastic cells.

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Key words: tropomyosin; chimeras; stress fibers; anchorage independent growth; cytokinesis.

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Abstract:

The presence of aberrant cytoskeleton, arising from the downregulation of key cytoskeletal proteins such as tropomyosins (TMs), is a prominent feature of many malignant cells and is suggested to promote neoplastic growth. While our previous work demonstrated that tropomyosin-1 (TM1) promotes stress fiber assembly and suppresses malignant growth, the molecular basis of the anti-oncogenic effects of TM1 has not been determined. By employing chimeric TMs, here we demonstrate that the amino terminal portion of TM1, but not the carboxy terminal portion which contains the alternatively spliced exon-coded sequences, is essential for stress fiber assembly and suppression of malignant growth. These studies also indicate that the amino and carboxy termini of TM1 coordinately function to regulate microfilament organization during cytokinesis.

1. **Introduction**:

The polymerization status of actin is dynamically regulated by a number of actin binding proteins which modulate the cytoskeletal organization in a spatio-temporal manner [1]. In cultured fibroblasts, actin cytoskeleton is organized into filopodia [2], lamellipodia [3] and stress fibers [4]. Stress fibers are bundles of microfilaments that traverse the cells and their absence contributes to the aberrant cytoskeleton and deranged cellular morphology often associated with the neoplastic cells [5-8]. Stress fiber assembly is regulated by RhoA-mediated contractility in normal cells [9]. Several studies have shown that the active RhoA in malignant cells is uncoupled from its downstream effectors, and is suggested as a mechanism for the presence of disorganized cytoskeleton in neoplastic cells [10-13].

The expression of key actin binding proteins, such as α-actinin, vinculin, gelsolin, caldesmon and tropomyosins (TMs) is downregulated in neoplastic cells (reviewed in [14-16]). We propose that the downregulation of actin binding proteins is responsible for the dissolution of stress fibers, resulting in aberrant cytoskeleton, altered cellular morphology, and enhanced cell motility and invasion. Such an idea is supported by several studies in which the restoration of α-actinin [7], vinculin [8] and TMs [6, 17-19] results in the re-emergence of stress fibers and suppression of malignant growth.

Tropomyosin (TM) family of actin binding proteins is of particular interest because of the consistent loss of certain isoforms, such as tropomyosin-1 (TM1) isoform, in diversely transformed cells. TM1 is downregulated in primary breast tumors [20] and transitional carcinoma of the urinary bladder [21]. The expression of TM1 is consistently abolished in breast carcinoma cell lines [19, 22], and is downregulated in a several

melanoma, colon and lung carcinoma cell lines (unpublished data) through epigenetic mechanisms [23, 24]. Previous studies from this laboratory have shown that TM1 induces anoikis [20] and suppresses the neoplastic growth of several malignant cells [6, 17-19]. Whereas some reports indicate that other TMs also might reorganize cytoskeleton and suppress malignant growth [25-27], our results demonstrate that the anti-oncogenic properties are specific for TM1 [17, 20] and the potential reasons for the apparent variance have been discussed else where [28].

The molecular basis for TM1-mediated cytoskeletal and anti-oncogenic effects is intriguing and remains to be elucidated. All TMs share extensive sequence homology, yet they differ remarkably in their expression in tissues through alternative splicing and in subcellular localization [14, 29]. In vitro studies have shown that the amino and carboxy termini of TM proteins are key determinants of actin-binding properties of various TMs and hence may specify the isoform-specific functions [30]. In this report, we have determined the structural components of TM1 that mediate cytoskeletal reorganization and suppression of neoplastic growth by utilizing chimeras of TM1 and TM2. Our studies also indicate that distinct sequences ('domains') of TMs perform specific functions in microfilament organization.

2. Materials and Methods:

2.1. Plasmids and TM constructs

To construct chimeras of TM1 [31] and TM2 [17], a Hind III site was engineered into TM1 and TM2 cDNAs by PCR based silent mutagenesis at nucleotide 506 (G →T) of the coding region, with 'A' of the initiation codon being position 1. The amino and carboxy

portions of TM1 and TM2 were interchanged at the Hind III site by standard molecular biology procedures, as illustrated in Figure 1. The TM2-TM1 chimera was subcloned into a ptTAs vector (a generous gift from Dr. E. P. Reddy, Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine) that combines the sequences of the tetracycline controlled transactivator (tTA) and the tet operator-CMV-promoter into a single plasmid. The TM1-TM2 chimera was subcloned into pIRES2-EGFP vector (Clontech) as an EcoR I-BamH I fragment that co-expresses GFP as a visual marker. The nucleotide sequence of the chimeras was verified by sequencing. The constructs are expected to encode 284 amino acid- containing chimeric proteins.

2.2. Cell culture and transfection

NIH3T3 cells transformed by *v-Ki-ras* (DT cells) and the cell lines expressing TM1 (DT/TM1) have been described previously [6, 32]. DT cells express TM1 at the 50% levels found in NIH3T3 cells, whereas TM2 and TM3 levels are undetectable. Transduction of TM1 markedly enhances TM1 expression in DT cells [6]. The DT cells were transfected with a recombinant TM2-TM1 plasmid, selected for resistance to puromycin and cloned by limiting dilution method in the presence of tetracycline (2μg/ml). DT cells were also transfected with an empty ptTAs vector to generate vector control cells for the experiments involving DT/TM2-TM1 cells.

Cells transfected with TM1-TM2 chimera were selected for G418 resistance and sorted by flow cytometry to isolate transfected cells. Cells transfected with empty vector (pIRES2-EGFP) were also processed similarly for use as controls.

2.3 Other methods

Cell lysates were prepared as described previously [33] and analyzed by immunoblotting using 50 μg of total cytoplasmic proteins. Immunofluorescence was performed using TM311 monoclonal antibody (Sigma), which recognizes all TMs expressed in this cell type [33]. Anchorage independent growth was measured by soft agar assays [6]. Flow sorting of transfected cells was performed using Beckton Dickinson FACStar Plus Flow cytometer.

3. Results and Discussion:

Many cytoskeletal proteins involved in regulating microfilament dynamics contain various structural motifs that mediate specific functions. TM1, like the other TMs, is an actin binding protein with no defined catalytic activity or specific binding partners that could regulate intracellular signaling pathways to mediate the isoform-specific functions of TM1. However, several in vitro studies suggest that the amino and carboxy termini are important determinants of TM functions [30, 34]. Furthermore, studies with transgenic mice expressing variants of cardiac TM isoforms have demonstrated that the carboxy termini TMs are important for the cardiac muscle function [35, 36].

The high M_r TMs (TM1, TM2 and TM3) consist of 284 amino acids (coded by 9 exons), share extensive sequence homology, localize to stress fibers and bind to actin at 1:7 molar ratio (reviewed in [14, 29]). We have utilized TM2 to determine whether other TM isoforms also mimic the anti-oncogenic effects of TM1, and have shown that TM2 fails to induce stress fibers and suppress anchorage-independent growth in highly malignant DT (*ras*-transformed NIH3T3) cells [17] and MCF-7 breast carcinoma cells

[20]. TM1 and TM2 share extensive sequence identity (85.6%) and many other features (Figure 1). For example, both proteins are α-helical coiled coil proteins (reviewed in [14]) and form homodimers [28]. However, TM1 and TM2 diverge significantly in carboxy terminal sequences encoded by exons 6 (52% sequence identity) and exon 9 (69% sequence identity), suggesting that these regions may mediate TM1-specific functions. The alternatively spliced exons of TMs have been implicated in isoform-specific functions such as modulating the interaction with troponin complex during muscle contraction [37]. Therefore, we created chimeras of TM1 and TM2, as described in Methods, and expressed them in DT cells.

3.1. TM2-TM1 chimera fails to induce stress fibers and suppress malignant growth

Stable cell lines of DT cells transfected with TM2-TM1 chimera (DT/TM2-TM1) were isolated. The expression of the chimera was induced to about 10-fold in the absence of tetracycline (Figure 2A). Expression of endogenous TM1 is not altered when the chimera is induced. TM1 levels in the chimera transfected cells are comparable, in presence or absence of the antibiotic, to the level found in DT cells, as measured by normalization to tubulin load control (data not shown). Expression of TM2-TM1 chimera in DT cells failed to reorganize microfilaments, assemble stress fibers and consequently did not alter the cell morphology (Figure 2B). Further, induction of DT/TM2-TM1 chimera did not inhibit the anchorage independent growth of DT cells (Figure 2C). DT/TM2-TM1 cells grew equally efficiently under induced and repressed conditions in soft agar and the efficiency of colony formation was comparable to that obtained with the wild type DT cells. Expression of wild type TM1, however, nearly abolished anchorage independent growth. Collectively, these results show that TM2-

TM1 chimera, like TM2, is not a suppressor of malignant phenotype, indicating that the carboxy terminal portion of TM1 lacks the ability to reorganize cytoskeleton and confer tumor suppression.

3.2. The TM1-TM2 chimera induces stress fibers in DT cells

Since the amino terminal integrity is an important determinant of TM functions [33, 38], we have considered the possibility that a TM1-TM2 chimera may be able to mimic TM1's ability to reorganize microfilaments . Repeated attempts at generating stable clones that express TM1-TM2 chimera in several expression vectors by conventional drug selection strategies have been unsuccessful (data not shown). Therefore, the TM1-TM2 chimera was cloned into a plasmid vector that co-expressed enhanced green fluorescent protein (EGFP) from IRES sequences. DT cells transfected with the TM1-TM2 chimera were sorted out by flow cytometry for EGFP expression, with empty vector transfected cells serving as controls.

The flow sorted DT/TM1-TM2 chimera cells expressed the TM1-TM2 chimera, as determined by immunoblotting (Figure 3A). Morphologically, the chimera transfected cells grew as flat and well-spread cells while the vector transfected cells remained spindle shaped, reminiscent of DT cells. Examination of microfilament architecture revealed that DT/TM1-TM2 cells displayed well organized stress fibers that co-stain for TM and F-actin (Figure 3B). Vector control cells, however, resembled the parental DT cells and did not contain well organized stress fibers. The microfilament organization in DT/TM1-TM2 cells resembled that of DT/TM1 cells and further supports our findings that the amino terminal sequences of TM1 are important for its cytoskeletal functions. Whereas our previous work showed that the disruption of the amino terminal integrity with the

addition of a hemagglutin epitope (HA) tag abrogates stress fiber formation in malignant cells [33], the present work demonstrated that the amino terminal sequences of TM1 induce stress fibers. Together, these findings show that the amino terminal sequences of TM1 and the amino terminal integrity are critical for stress fiber assembly.

3.3. TM1-TM2 chimera induces defective cytokinesis

The flow sorted DT/TM1-TM2 cells contain a number of multinucleated cells (Figure 3C). Therefore, the expression of TM1-TM2 chimera results in a defective cytokinesis. The unusually high number of binucleated cells (> 25%) rendered the isolation of single cell clones of DT/TM1-TM2 cells, not possible. Furthermore, cells that survived and grew from these primary flow-sorted cultures did not express TM1-TM2 chimera and reverted to DT-like growth characteristics with spindle-shaped cell morphology. These 'revertant' cells, although retained the GFP expression, were lacking the expression of the TM1-TM2 chimera (not shown). These findings show that although TM1-TM2 chimera is able to promote stress fiber assembly, it induces abnormal cytokinesis indicating that the mismatched ends of TMs exert deleterious effect on growth.

High M_r TMs localize to the cytokinetic ring [39]. Previous work from this and other laboratories has shown that modification of the ends of TMs promotes defective cytokinesis. For instance, chimeras of TM5-TM3 which contains the amino terminal portion of TM5 (a low M_r TM) and the carboxy terminal half of TM3 (a high M_r TM), have been reported to display high actin affinity, abnormally high multinucleated cells and a delayed cytokinesis [40, 41]. Our recent work with the HA-TM1 also support that the ends of TMs are important regulators of TM functions and influence cytokinesis [33].

The mismatching of TM ends, in the case of TM1-TM2 chimera, however results in extensive endoreduplication and interferes with cytokinesis, which precluded detailed analyses of the block in cytokinesis. Collectively, these observations suggest that the coordinate regulation of the amino and carboxy termini of high M_r TMs is essential for normal cytokinesis.

The association of TMs, in particular TM1, with F-actin is regulated by interactions with other TMs such as TM2 and caldesmon [42]. Further, caldesmon is phosphorylated by p34cdc-2 kinase in a mitosis specific fashion, which results in a dissociation of caldesmon from the microfilaments [43]. The absence of caldesmon in microfilaments is suggested to weaken TM1-F-actin interactions, resulting in the dissociation of TM1 from microfilaments [42]. Additional studies will be necessary to determine whether the chimeras differ in their binding properties to actin and/or regulation by caldesmon.

In summary, our results show that the amino terminal portions of TM1 are critical determinants of TM1 in stress fiber assembly, cytokinesis and suppression of malignant growth. The carboxy terminus of TM1 is not sufficient to induce stress fibers, and may co-ordinate the binding interactions with F-actin or contributes to the other anti-oncogenic properties of TM1. Thus, the microfilament organization and the induction of stress fiber assembly are important factors in the anti-neoplastic actions of TM1.

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References:

[1] C. G. Dos Remedios, D. Chhabra, M. Kekic, I. V. Dedova, M. Tsubakihara, D. A. Berry, N. J. Nosworthy. Actin Binding Proteins: Regulation of Cytoskeletal Microfilaments, Physiol. Rev. 83 (2003) 433-473.

[2] W. Wood, P. Martin. Structures in focus--filopodia, Int. J. Biochem & Cell Biol. 34 (2002) 726-730.

[3] J. V. Small, T. Stradal, E. Vignal, K. Rottner. The lamellipodium: where motility begins, Trends Cell Biol. 12 (2002) 112-120.

[4] K. Katoh, Y. Kano, M. Masuda, H. Onishi, K. Fujiwara. Isolation and contraction of the stress fiber, Mol. Biol. Cell 9 (1998) 1919-1938.

[5] F. Matsumura, J. J. Lin, S. Yamashiro-Matsumura, G. P. Thomas, W. C. Topp. Differential expression of tropomyosin forms in the microfilaments isolated from normal and transformed rat cultured cells, J. Biol. Chem. 258 (1983) 13954-13964.

[6] G. L. Prasad, R. A. Fuldner, H. L. Cooper. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene, Proc. Natl. Acad. Sci. USA 90 (1993) 7039-7043.

[7] U. Gluck, D. J. Kwiatkowski, A. Ben-Ze'ev. Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with alpha-actinin cDNA, Proc. Natl. Acad. Sci. USA 90 (1993) 383-387.

[8] J. L. Rodriguez Fernandez, B. Geiger, D. Salomon, I. Sabanay, M. Zoller, A. Ben-Ze'ev. Suppression of tumorigenicity in transformed cells after transfection with vinculin cDNA, J. Cell Biol. 119 (1992) 427-438.

[9] M. Chrzanowska-Wodnicka, K. Burridge. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions, J. Cell Biol. 133 (1996) 1403-1415.

[10] I. Izawa, M. Amano, K. Chihara, T. Yamamoto, K. Kaibuchi. Possible involvement of the inactivation of the Rho-Rho-kinase pathway in oncogenic Ras-induced transformation, Oncogene 17 (1998) 2863-2871.

[11] E. Sahai, M. F. Olson, C. J. Marshall. Cross-talk between Ras and Rho signalling pathways in transformation favours proliferation and increased motility, EMBO J. 20 (2001) 755-766.

[12] G. Pawlak, D. M. Helfman. MEK Mediates v-Src-induced Disruption of the Actin Cytoskeleton via Inactivation of the Rho-ROCK-LIM Kinase Pathway, J. Biol. Chem. 277 (2002) 26927-26933.

[13] G. Pawlak, D. M. Helfman. Post-transcriptional down-regulation of ROCKI/Rho-kinase through an MEK-dependent pathway leads to cytoskeleton disruption in Ras-transformed fibroblasts, Mol Biol Cell 13 (2002) 336-347.

[14] J. J. Lin, K. S. Warren, D. D. Wamboldt, T. Wang, J. L. Lin. Tropomyosin isoforms in nonmuscle cells, Int. Rev. Cytol. 170 (1997) 1-38.

[15] A. Ben-Ze'ev. Cytoskeletal and adhesion proteins as tumor suppressors, Curr. Opin. Cell Biol. 9 (1997) 99-108.

[16] G. Pawlak, D. M. Helfman. Cytoskeletal changes in cell transformation and tumorigenesis, Curr. Opin. Gen. & Dev. 11 (2001) 41-47.

[17] R. H. Braverman, H. L. Cooper, H. S. Lee, G. L. Prasad. Anti-oncogenic effects of tropomyosin: isoform specificity and importance of protein coding sequences, Oncogene 13 (1996) 537-545.

[18] G. L. Prasad, L. Masuelli, M. H. Raj, N. Harindranath. Suppression of src-induced transformed phenotype by expression of tropomyosin-1, Oncogene 18 (1999) 2027-2031.

[19] K. Mahadev, G. Raval, S. Bharadwaj, M. C. Willingham, E. M. Lange, B. K. V. Vonderhaar, D. Salomon, G. L. Prasad. Suppression of the transformed phenotype of breast cancer by tropomyosin-1, Exp. Cell. Res. 279 (2002) 40-51.

[20] G. N. Raval, S. Bharadwaj, E. A. Levine, M. C. Willingham, R. L. Geary, T. Kute, G. L. Prasad. Loss of expression of tropomyosin-1, a novel class II tumor suppressor that induces anoikis, in primary breast tumors, Oncogene 22 (2003) 6194-6203.

[21] G. Pawlak, T. W. McGarvey, T. B. Nguyen, J. E. Tomaszewski, R. Puthiyaveettil, S. B. Malkowicz, D. M. Helfman. Alterations in tropomyosin isoform expression in human transitional cell carcinoma of the urinary bladder, Int J Cancer 110 (2004) 368-373.

[22] B. Bhattacharya, G. L. Prasad, E. M. Valverius, D. S. Salomon, H. L. Cooper. Tropomyosins of human mammary epithelial cells: consistent defects of expression in mammary carcinoma cell lines, Cancer Res. 50 (1990) 2105-2112.

[23] S. Bharadwaj, G. L. Prasad. Tropomyosin-1, a novel suppressor of cellular transformation is downregulated by promoter methylation in cancer cells, Cancer Lett. 183 (2002) 205-213.

[24] J. M. Shields, H. Mehta, K. Pruitt, C. J. Der. Opposing roles of the extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades in Ras-mediated downregulation of tropomyosin, Mol. Cell. Biol 22 (2002) 2304-2317.

[25] K. Takenaga, A. Masuda. Restoration of microfilament bundle organization in v-raf-transformed NRK cells after transduction with tropomyosin 2 cDNA, Cancer Lett. 87 (1994) 47-53.

[26] R. A. Janssen, J. W. Mier. Tropomyosin-2 cDNA lacking the 3' untranslated region riboregulator induces growth inhibition of v-Ki-ras-transformed fibroblasts, Mol. Biol. Cell 8 (1997) 897-908.

[27] M. Gimona, J. A. Kazzaz, D. M. Helfman. Forced expression of tropomyosin 2 or 3 in v-Ki-ras-transformed fibroblasts results in distinct phenotypic effects, Proc. Natl. Acad. Sci. USA 93 (1996) 9618-9623.

[28] V. Shah, R. Braverman, G. L. Prasad. Suppression of neoplastic transformation and regulation of cytoskeleton by tropomyosins, Somat Cell Mol Genet 24 (1998) 273-280.

[29] M. F. Pittenger, J. A. Kazzaz, D. M. Helfman. Functional properties of non-muscle tropomyosin isoforms, Curr. Opin. Cell Biol. 6 (1994) 96-104.

[30] J. Moraczewska, K. Nicholson-Flynn, S. E. Hitchcock-DeGregori. The ends of tropomyosin are major determinants of actin affinity and myosin subfragment 1-induced binding to F-actin in the open state, Biochemistry 38 (1999) 15885-15892.

[31] G. L. Prasad, P.S.Meissner, D.Sheer, H.L.Cooper. A cDNA encoding a muscle-type tropomyosin cloned from a human epithelial cell line: Identity with human fibroblast tropomyosin, TM1, Biochem. Biophys. Res. Commun. 177 (1991) 1068-1075.

[32] V. Shah, S. Bharadwaj, K. Kaibuchi, G. L. Prasad. Cytoskeletal organization in tropomyosin-mediated reversion of ras-transformation: Evidence for Rho kinase pathway, Oncogene 20 (2001) 2112-2121.

[33] S. Bharadwaj, S. Hitchcock-DeGregori, A. Thorburn, G. L. Prasad. N Terminus Is Essential for Tropomyosin Functions: N-TERMINAL MODIFICATION DISRUPTS STRESS FIBER ORGANIZATION AND ABOLISHES ANTI-ONCOGENIC EFFECTS OF TROPOMYOSIN-1, J. Biol. Chem. 279 (2004) 14039-14048.

[34] Y. J. Cho, J. Liu, S. E. Hitchcock-DeGregori. The amino terminus of muscle tropomyosin is a major determinant for function, J. Biol. Chem. 265 (1990) 538-545.

[35] G. Jagatheesan, S. Rajan, N. Petrashevskaya, A. Schwartz, G. Boivin, S. Vahebi, P. DeTombe, R. J. Solaro, E. Labitzke, G. Hilliard, D. F. Wieczorek. Functional Importance of the Carboxyl-terminal Region of Striated Muscle Tropomyosin, J. Biol. Chem. 278 (2003) 23204-23211.

[36] R. D. Gaffin, K. Gokulan, J. C. Sacchettini, T. Hewett, R. Klevitsky, J. Robbins, M. Muthuchamy. Charged residue changes in the carboxy-terminus of {alpha}-tropomyosin alter mouse cardiac muscle contractility, J Physiol (Lond) 556 (2004) 531-543.

[37] B. M. Wolska, D. M. Wieczorek. The role of tropomyosin in the regulation of myocardial contraction and relaxation, Pflugers Arch 446 (2003) 1-8.

[38] J. H. Brown, K. H. Kim, G. Jun, N. J. Greenfield, R. Dominguez, N. Volkmann, S. E. Hitchcock-DeGregori, C. Cohen. Deciphering the design of the tropomyosin molecule, Proc. Natl. Acad. Sci. USA 98 (2001) 8496-8501.

[39] J. A. Hughes, C. M. Cooke-Yarborough, N. C. Chadwick, G. Schevzov, S. M. Arbuckle, P. Gunning, R. P. Weinberger. High-molecular-weight tropomyosins localize to the contractile rings of dividing CNS cells but are absent from malignant pediatric and adult CNS tumors, Glia 42 (2003) 25-35.

[40] K. S. Warren, J. L. Lin, J. P. McDermott, J. J. Lin. Forced expression of chimeric human fibroblast tropomyosin mutants affects cytokinesis, J. Cell Biol. 129 (1995) 697-708.

[41] K. Wong, D. Wessels, S. L. Krob, A. R. Matveia, J. L. Lin, D. R. Soll, J. J. Lin. Forced expression of a dominant-negative chimeric tropomyosin causes abnormal motile behavior during cell division, Cell Motil Cytoskeleton 45 (2000) 121-132.

[42] M. F. Pittenger, A. Kistler, D. M. Helfman. Alternatively spliced exons of the beta tropomyosin gene exhibit different affinities for F-actin and effects with nonmuscle caldesmon, J. Cell. Sci. 108 (1995) 3253-3265.

[43] S. Yamashiro, Y. Yamakita, H. Hosoya, F. Matsumura. Phosphorylation of non-muscle caldesmon by p34cdc2 kinase during mitosis, Nature 349 (1991) 169-172.

Figure Legends

Figure 1. Generation of TM chimeras: A schematic representation of TM1 and TM2 cDNAs with areas of sequence divergence, and the position at which the amino and carboxy termini are switched (designated as 'Switch') is depicted. The TM2-TM1 chimera is subcloned into a tetracycline regulatable plasmid vector, ptTAs that allows selection with puromycin. In this vector, the tetracycline regulated transactivator and the tetracycline-repressed CMV promoters are present. The expression of the cloned gene is repressed in the presence of the antibiotic. The TM1-TM2 chimera is cloned into pIRES2-EGFP plasmid, and the transfected cells are isolated by flow cyotometer.

Figure 2: TM2-TM1 chimera does not assemble stress fibers or suppress anchorage-independent growth: ***A.*** DT/TM2-TM1 cell line was tested for the expression of the chimera by immunoblotting with TM311 antibody (Sigma) in the presence and the absence of tetracycline (Tet), and the chimera is identified. For control purposes, NIH3T3 and DT cells were used. The top band in NIH3T3 cells is TM1; whereas the lower band corresponds to a mixture of TM2 and TM3 isoforms, which are resolved on 2-D gels. The DT/TM2-TM1 samples were under-exposed, as the chimera is induced to ten fold in the absence of the antibiotic. Therefore, the TM1 signal is not evident under these conditions. ***B.*** Immunofluorescence revealed that the induction of TM2-TM1 chimera fails to induce stress fibers, as revealed by TM antibody and F-actin (staining with phalloidin), and the cells resembled parental DT and DT/TM2 cells, as shown previously [17, 28, 32, 33]. Bar 10μm. ***C.*** Expression of the TM2-TM1 chimera does not inhibit the anchorage independent growth. One thousand cells were plated on soft

agar and the number of colonies were enumerated at the end of 2 weeks following staining with 0.5% nitro blue tetrazolium. The DT/TM2-TM1 cell line was cultured either in the presence of tetracycline (+) or absence (-), which corresponds to uninduced and induced conditions, respectively. A vector control cell line (DT/V) was also plated which efficiently grew under anchorage-independent conditions. The number of colonies obtained is normalized to DT cells (100%). Photomicrographs of soft agar cultures (X10 magnification) are shown below the graph. TM1 expression (DT/TM1 cells), abolishes the growth of DT cells and is included as a reference [6, 17]. Expression of TM2 in DT cells does not alter anchorage independent growth [17], and therefore, not depicted.

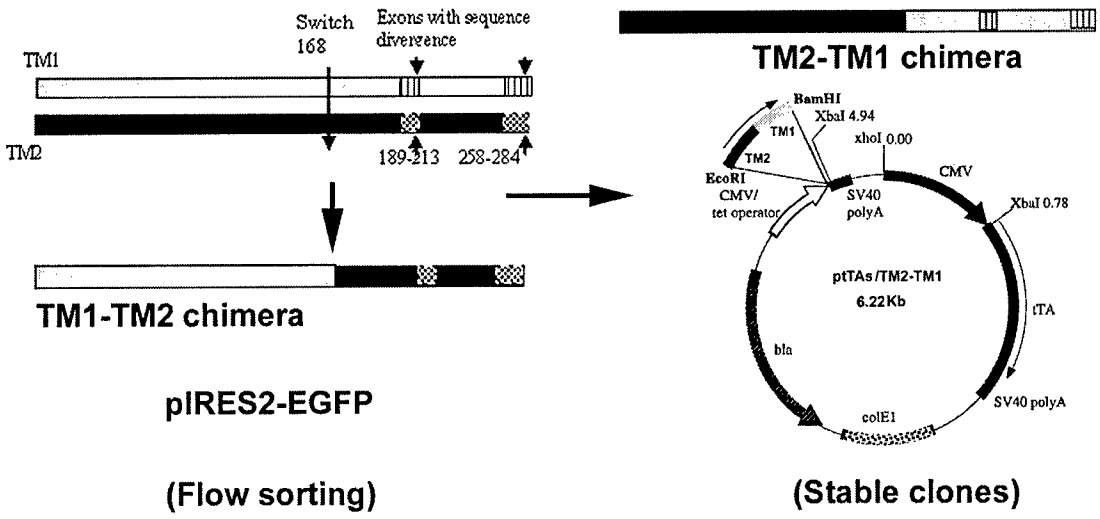
Figure 3. TM1-TM2 chimera induces stress fiber formation: ***A***. The flow sorted DT/TM1-TM2 chimera cells were tested for the expression of the chimera by immunoblotting using TM311 antibody. The empty vector transfected cells (labeled DT/vector), and the chimera transfected cells (labeled DT/chimera) were analyzed. The position of TM1-TM2 chimera is identified.

B. DT cells transected with empty pIRES2-EGFP (vector control) and the chimera (TM1-TM2 chimera) cells were stained with TM311 antibody and phalloidin. Expression of TM1-TM2 chimera results in the assembly of stress fibers that are stained with TM antibody and phalloidin. The inset in the TM1-TM2 chimera cells (TM- panel) shows the presence of organized bundles of microfilaments at a higher magnification. The stress fibers in the chimera transfected cells costain with TM antibody and phalloidin and traverse the cell (also seen in panel C). The vector control cells lack TM-positive-

microfilaments and resemble the parental DT cells. Both cell types are positive for GFP, as indicated. Bar 10μm.

C. The DT/TM1-TM2 cells display defective cytokinesis. The flow sorted DT/TM1-TM2 cells were stained as indicated. The arrows show binucleated cells. Bar 10 μm.

Figure 1



pIRES2-EGFP

(Flow sorting)

(Stable clones)

Figure 2

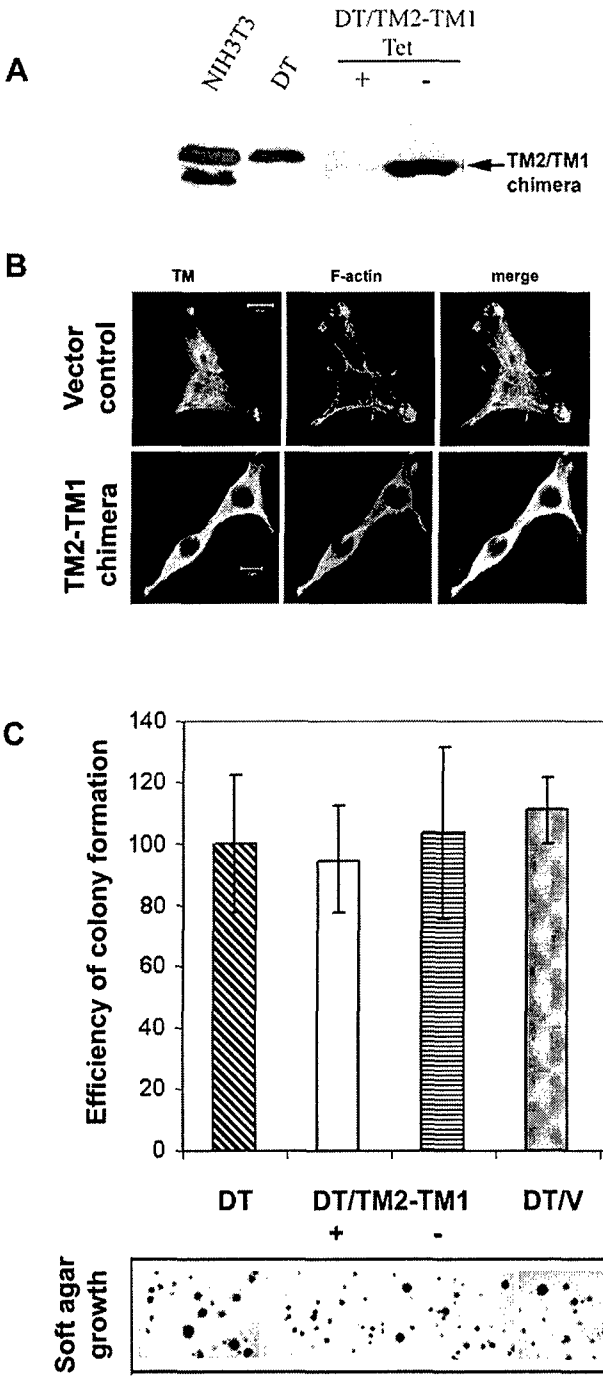
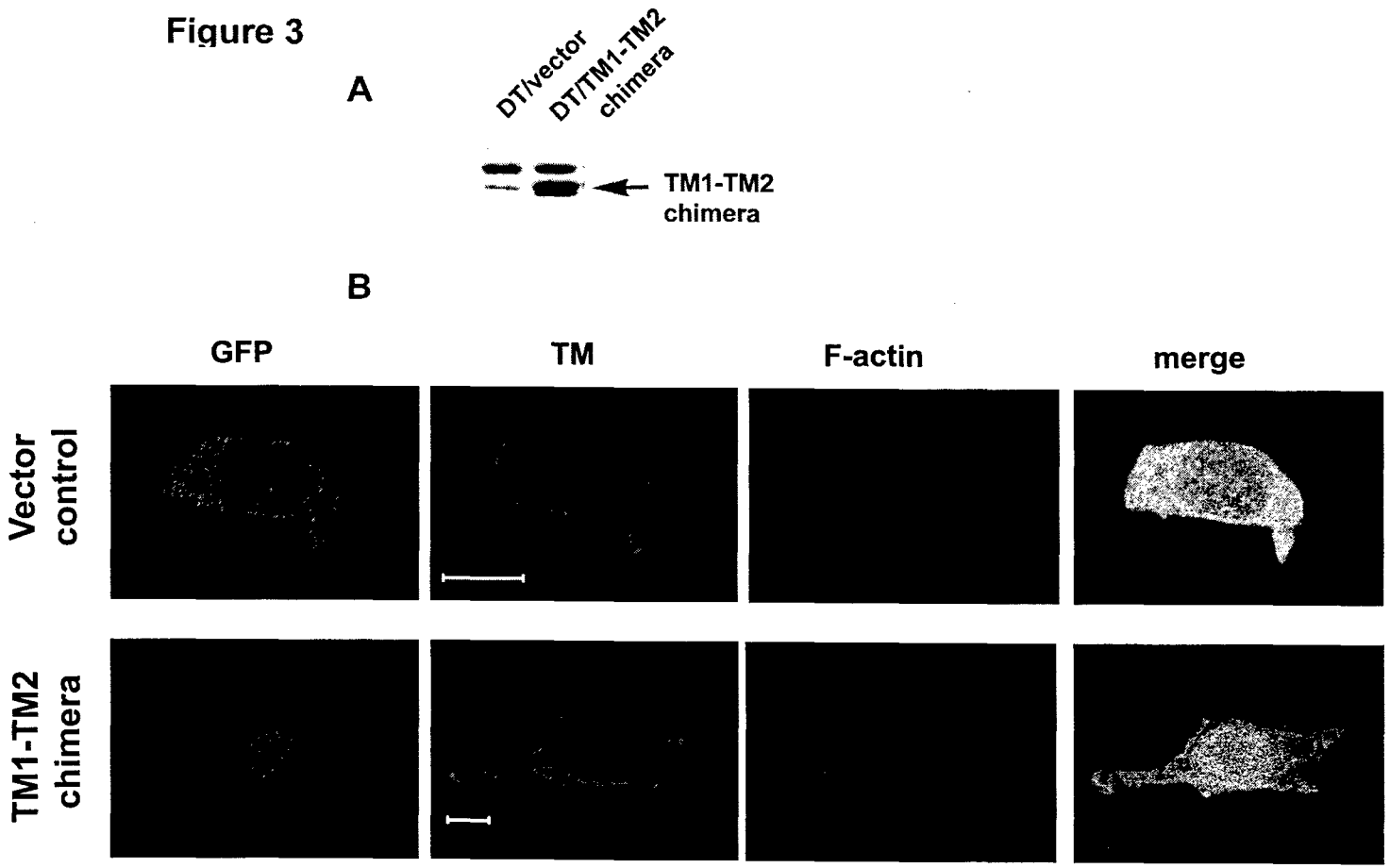

A
NIH3T3
DT
DT/TM2-TM1
Tet
+
-
TM2/TM1
chimera
B
TM
F-actin
merge
Vector
control
TM2-TM1
chimera
C
Efficiency of colony formation
140
120
100
80
60
40
20
0
DT
DT/TM2-TM1
+
-
DT/V
DT/TM1
Soft agar
growth

Figure 3


A
DT/vector
DT/TM1-TM2 chimera
TM1-TM2 chimera
B
GFP
TM
F-actin
merge
Vector control
TM1-TM2 chimera
C
TM
F-actin
DT/TM1-TM2

APPENDIX 8

Amino terminal region is essential for tumor suppression by Tropomyosin-1, a novel class II tumor suppressor.

Shantaram Bharadwaj, Gaddamanugu L. Prasad, Wake Forest University Health Sciences, Winston-Salem, NC.

Abstract – AACR-7/11/03 - 7/14/03: Washington D.C

Microfilaments regulate a number of cellular processes such as cytokinesis, motility and intracellular transport, and are critical for maintaining normal cell morphology. Disorganized microfilaments are generally found in malignant cells and are thought to play a crucial role in neoplastic transformation of cells. Tropomyosin-1 (TM1) is a key actin binding protein that stabilizes microfilaments, and suppression of TM1 expression is a common biochemical change in malignant cells. Previous work from this laboratory demonstrates that TM1 is a novel class II tumor suppressor, and that TM1 is profoundly downregulated in breast carcinomas. The present study investigates the structure-function relationship of tumor suppression by TM1.

TM1 undergoes dimerization and associates with F-actin in a head-to-tail fashion, thus spanning the entire length of the filament. Consistently, amino and carboxy termini of TM1 have been shown to be important determinants of TM1 for binding to actin. In vitro binding studies have shown that modification of the N-terminus of TM1 results in decreased binding to actin, which could impair the assembly and the stability of actin filaments. Therefore, we modified the amino terminal end of TM1 by addition of a HA epitope. We have utilized DT cells (ras transformed NIH3T3 cells) which express significantly lower TM1 compared with the parental NIH3T3 cells.

Stable cell lines of DT expressing the variant TM1 (DT/HA-TM1) have been generated, and their growth characteristics were compared with DT cells expressing wild type TM1 (DT/TM1). Expression of HA-TM1 protein did not alter cellular morphology, growth rates in monolayer cultures or induce microfilaments. In contrast, wild type TM1 significantly decreased the growth rates and reorganized cytoskeleton leading to improved cellular morphology. Furthermore, the variant TM1 protein did not alter the anchorage independent growth –an important property of malignantly transformed cells– , while the wild type TM1 abolished anchorage independent growth.

We investigated if the inability of HA-TM1 protein to suppress malignant transformation is due to prevention of dimerization. Cross linking experiments reveal that HA-TM1 does not form dimers, while the wild type protein exists as dimers and associates with the detergent insoluble fraction (cytoskeletal compartment). To determine whether the variant protein binds to F-actin, NIH3T3 cells were transfected with HA-TM1. The variant protein colocalizes with stress fibers indicating that the failure to suppress transformed growth is not due to the inability of mutant protein to bind to F-actin.

These data suggest that the amino terminus region of TM1is important for tumor suppression. Further experiments are in progress to determine how amino terminal modification affects cytoskeletal organization and tumor suppression by TM1.

Cytoskeletal Proteins as Regulators of Breast Cancer: Anoikis and Tumor Suppression by Tropomyosin-1, a Microfilament Stabilizing Protein.

Shantaram Bharadwaj, Edward A Levine and G. L. Prasad

While surgery remains as the most effective means to control primary breast cancer, most fatalities in breast cancer patients occur due to the metastatic disease. Two most common properties of malignant cells are the presence of disorganized cytoskeleton and the ability to grow at non-physiological loci. For example, it is well established that cytoskeleton regulates cell morphology, cell division and cell motility. Cytoskeletal disorganization, caused by changes in the expression of cytoskeletal proteins during neoplastic transformation, leads to deregulation of these processes and facilitates invasion and malignant growth. Second, normal cells undergo anoikis (detachment-induced apoptosis) when removed from extracellular matrix (ECM). In contrast, tumor cells are resistant to anoikis and proliferate in the absence of adhesion-derived survival signals, as metastases. Therefore, investigations into mechanisms that promote metastasis may lead to better options to treat breast cancer.

Earlier work from this laboratory has identified the loss of expression of tropomyosin isoform 1 (TM1)—a microfilament stabilizing protein— as a common biochemical change in the breast cancer cell lines. Consistent downregulation of TM1 appears to be specific for breast cancer cells, as about 50% lung and colon carcinoma cells do contain significant levels of TM1. TM1 expression is extinguished in breast cancer cells by epigenetic mechanisms involving histone deacetylation and gene methylation. Restoration of expression of TM1 in two widely studied breast cancer cell lines results in reorganization of actin cytoskeleton. Significantly TM1 also inhibits the malignant growth of breast cancer cell lines, independent of p53 or estrogen receptor status. The anti-neoplastic effects of TM1 are isoform specific; other closely related TMs do not affect the malignant growth properties of breast cancer cells. Collectively these results suggest that suppression of TM1 may be a key event during mammary carcinogenesis, and that TM1 is a class II tumor suppressor.

To investigate the significance of the lack of TM1 expression in the established breast cancer cell lines, we have assessed changes in TM1 expression in 25 normal and 25 breast tumor tissue specimens collected at the Wake Forest University School of Medicine. By *in situ* hybridization and immunofluorescence, TM1 expression is readily detectable in the normal ductal epithelium, but undetectable in the tumor tissues. These results, for the first time, demonstrate that the loss of TM1 expression is a common biochemical event in breast cancer.

Investigations into TM1-induced tumor suppression suggest that TM1 induces anoikis in breast cancer cells. While several proteins such as integrins and focal adhesion kinases are known to regulate adhesion-dependent survival, modulation of anoikis by a structural, microfilament protein is novel. Thus, the loss of TM1 expression during the neoplastic transformation contributes to loss of cellular morphology, and may confer resistance to anoikis. Ongoing work addresses the detailed mechanism of TM1-induced anoikis. Because TM1 suppresses the basic mechanisms that are essential for metastasis, it is likely these investigations may identify novel pathways/targets amenable for drug development.

Gordon Conference on Signaling by Adhesion Receptors. (June 04, Bristol, RI).

Cytoskeletal Proteins as inducers of Anoikis: Studies on Tropomyosin-1-mediated integrin activity and Rho kinase signaling.

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ABSTRACT

Two most common properties of malignant cells are the presence of disorganized cytoskeleton and the ability to grow at non-physiological loci. For example, it is well established that cytoskeleton regulates cell morphology, cell division and cell motility. Cytoskeletal disorganization, caused by changes in the expression of cytoskeletal proteins during neoplastic transformation, leads to deregulation of these processes and facilitates invasion and malignant growth. Second, normal cells undergo anoikis (detachment-induced apoptosis) when removed from extracellular matrix (ECM). In contrast, tumor cells are resistant to anoikis and proliferate in the absence of adhesion-derived survival signals, as metastases.

We have utilized tropomyosin family of actin-binding proteins as a model to investigate the role of cytoskeleton in cell growth regulation. The loss of expression of tropomyosin isoform 1 (Tm1) occurs in breast and other neoplasms. Restoration of expression of TM1 in two widely studied breast cancer cell lines results in reorganization of microfilaments and suppression of neoplastic growth. Recently we showed that Tm1 induces anoikis (detachment-induced apoptosis) in breast cancer cells.

In the current study, we have investigated the mechanism of Tm1-induced anoikis in breast cancer cells.